Gene Expression Profiling

Microarrays were fabricated that contained 9,600 cDNA clones isolated from randomly collected from a normalized cDNA libraries or purchased from Research Genetics. Briefly, 9,600 cDNA inserts were generated by PCR amplification with primers derived from flanking

5   vector sequences, purified by gel filtration over Sephacryl 400 (Amersham), dried by lyophilization, and resuspended in 10 µl of 2X SSC. PCR products were arrayed from 96-well microtiter plates onto sialylated microscope slides in an area of 1.8 cm^2 using print tips constructed as elongate capillary channels, and driven by high-speed robotics. Fluorescently labeled probe pairs were applied to the microarray and allowed to hybridize to each of the 9,600

10   elements. Degree of hybridization at each element was quantified by sequential excitation of the 2 fluorophores with a scanning laser read at an appropriate wavelength for each emission. Differential expression values were expressed as a ratio of intensities from the two emissions where positive and negative values indicated an increase or decrease, respectively, relative to control. Expression data for particular target genes were rejected if neither channel produced a

15   signal at least 2.5-fold over local background or if the signal derived from less than 40% of the area of the printed spot.

Normal Rat Kidney Cells

Normal rat kidney cells (NRK) were cultured in DMEM-21 (high glucose)10% FCS at 37 °C, 5% CO2. Cells were serum starved for 24 hours, before treated with 5 ng/ml huTGF-β1

20   (R&D System) +/- inhibitors for additional 24 hours. Media were removed and cells were washed with PBS for total RNA extraction.

Results

Figure 5 shows the results of a representative microarray gene expression profiling analysis of cultured rat lung fibroblasts (RLF) and normal rat kidney cells (NRK). Expression of

25   the listed fibrotic genes was altered by TGF-β treatment at 24 hours and was reversed by co-treatment with certain TGF-β inhibitors selected from the compounds listed in the Tables above.

Figure 6 shows the results of a representative microarray gene expression profiling analysis and quantitative real time PCR of rat whole blood cells. TGF-β induces osteopontin gene expression at 4 hours and this induction is blocked by a representative of the TGFβ-R1

30   inhibitors of formula (1) listed in the Tables above.

Example 3

Effect of TGF-β inhibitors on profibrotic gene expression in the bleomycin rat model of pulmonary fibrosis

35   Material and Methods:

Animal information: 275-325 grams, male, Sprague-Dawley rats. Rats were anesthetized with 1.3 ml/kg of 0.8 mg/ml ketamine and 0.5 mg/ml xylazine cocktail. Once the rats were anesthetized, a 16G X 2" Surflo I.V. catheter was inserted into the trachea, 0.5 ml of saline or 0.5ml of 1.0 units/ml of bleomycin was slowly delivered into the lungs via a 8.5 cm polyethylene tubing PE-50 attached to a 23G X 1" needle which was connected to a 1.0 ml syringe. After the intratracheal administration of saline or bleomycin, two rats were housed in a new cage with new bedding and free access to food and water. Twenty four hours after the incubation, rats were weighted and orally dosed with 5 ml/kg of 1% methyl cellulose (MC) or 5 ml/kg of 2.0, or 6.0 mg/ml test compound twice a day for 4 and a half days or intraperitoneal injection of 2 ml/kg of 4.0 mg/ml of dexamethasone every other day for four and a half day. On day 1, 3, and 5, 400 μl of blood were collected from each rats via the tail to determine the circulation level of test compound. On day 5, rats were sacrificed and bronchoalveolar lavage fluids (BALF) were collected for protein (BCA Protein Assay Kit from Pierce (Cat #: 23225)) and interleukin 6 (R&D System Quantikine® M Rat IL-6 Immunoassay (Cat #: R6000)) analysis, and lung tissues were also collected for Taqman analysis. The test compound in these experiments is selected from Tables 1-5.

Results

The results are shown in Figures 7-13. Bleomycin (Bleo) administration increased interleukin-6 (IL-6) levels in bronchoalveolar lavage fluids (BALF) ($p < 0.001$) (Figure 9). BALF IL-6 levels were significantly decreased by the treatment of 30 mg/kg of the TGF-β inhibitor test compound ($p < 0.01$) or dexamethasone (Dex) ($p < 0.001$) (Figure 9). Furthermore, treatment of 30 mg/kg of the test compound, but not dexamethasone treatment, was associated with reduced levels of TGFβ-associated pulmonary mRNAs including PAI-1 ($p < 0.06$) (Figure 10), CTGF ($p < 0.01$) (Figure 11), TIMP-1 ($p < 0.1$) (Figure 12) and fibronectin ($p < 0.01$) (Figure 13).

In a similar experiment, synergistic effect of dexamethasone (Dex) and a representative test compound from compounds listed above in the bleomycin rat model of pulmonary fibrosis was studied. Rats were intubated with saline or 1 unit of bleomycin. After twenty four hours, rats were weighed and dosed with saline or 2.0 mg/kg of dexamethasone every other day and 1% methyl cellulose (MC) or 40 mg/kg of a test compound twice a day. On day 14, 1-3 hours after dosing, rat were sacrificed and lungs were inflated and collected for analysis.

These data support a synergistic effect between dexamethasone and the test compound. The primary end points of the analysis were the total hydroxyproline concentration per lung and lung capacity. The secondary end point was the body weight.

The results are shown in Figures 44-46. The statistical analysis was done using one-way ANOVA with Bonferroni's Multiple Comparison Test.

Figure 44 shows that bleomycin administration induces a significant body weight loss ($p<0.001$), while treatment with Dex, the test compound, or Dex combined with the test compound has no effect on the bleomycin-induced body weight loss.

Figure 45 shows that bleomycin administration induces a significant increase in total hydroxyproline in the lung ($p<0.001$). In addition, the Figure shows that the treatment with Dex ($p<0.05$), and Dex combined with the test compound ($p<0.001$) significantly decreases the total hydroxyproline concentration in the lung, induced by bleomycin. In particular, the treatment with Dex combined with the test compound shows significantly less total hydroxyproline in the lung than the treatment with Dex or the test compound alone ($p<0.001$).

Figure 46 shows that bleomycin administration induces a significant decrease in lung capacity ($p<0.001$). In addition, the Figure shows that the treatment with the test compound and Dex combined with the test compound significantly increases lung capacity which was reduced by bleomycin. In particular, the treatment with Dex combined with the test compound shows a significantly higher lung capacity than the lung capacity achieved by administration of Dex alone ($p<0.05$).

There is a trend in reducing bleomycin induced lung fibrosis with Dex (reduction in hydroxyproline, $p<0.05$), or with the test compound (increase in lung capacity, $p<0.05$) treatment alone. The combination treatment of Dex and the test compound significantly reduces bleomycin-induced lung fibrosis (reduction in hydroxyproline, and increase in lung capacity, $p<0.001$).

All references cited throughout the specification are expressly incorporated herein by reference. While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, and the like. All such modifications are within the scope of the claims appended hereto.

58

WHAT IS CLAIMED IS:

1   1.   A method for the treatment of a fibroproliferative disease, comprising

2   (a) identifying a non-peptide small molecule, selectively binding to a TGFβ-R1 kinase

3   receptor; and

4   (b) administering an effective amount of said molecule to a mammalian subject

5   diagnosed with said fibroproliferative disease.

1   2.   The method of claim 1 wherein said fibroproliferative disease is a renal, hepatic,

2   pulmonary, cardiovascular, eye, or dermatological disorder associated with enhanced TGF-β

3   activity and excessive fibrosis or sclerosis.

1   3.   The method of claim 1 wherein said fibroproliferative disease is selected from the

2   group consisting of glomerulonephritis (GN); diabetic nephropathy; renal interstitial fibrosis;

3   renal fibrosis resulting from complications of drug exposure; HIV-associated nephropathy;

4   transplant necropathy; liver cirrhosis due to all etiologies; disorders of the biliary tree; hepatic

5   dysfunction attributable to infections; pulmonary fibrosis; adult respiratory distress syndrome

6   (ARDS); chronic obstructive pulmonary disease (COPD); idiopathic pulmonary fibrosis (IPF);

7   acute lung injury (ALI); pulmonary fibrosis due to infectious or toxic agents; congestive heart

8   failure; dilated cardiomyopathy; myocarditis; vascular stenosis; progressive systemic sclerosis;

9   polymyositis; scleroderma; dermatomyositis; fascists; Raynaud's syndrome, rheumatoid arthritis;

10   proliferative vitreoretinopathy; fibrosis associated with ocular surgery; and excessive or

11   hypertrophic scar or keloid formation in the dermis occurring during wound healing resulting

12   from trauma or surgical wounds.

1   4.   The method of claim 1 wherein said molecule additionally inhibits a biological

2   activity mediated by p38 kinase.

1   5.   The method of claim 1 wherein said molecule preferentially inhibits a biological

2   activity mediated by TGF-β-RI kinase relative to a biological activity mediated by p38 kinase.
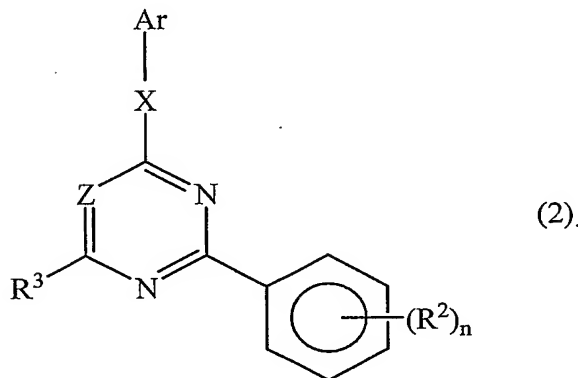
1   6.   The method of claim 1 wherein said molecule is a compound of formula (1)
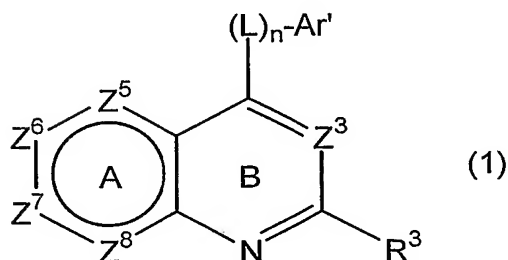
2

3

4   and the pharmaceutically acceptable salts and prodrug forms thereof

5   wherein R^3 is a noninterfering substituent;

6   each Z is CR^2 or N, wherein no more than two Z positions in ring A are N, and

7   wherein two adjacent Z positions in ring A cannot be N;

8   each R^2 is independently a noninterfering substituent;

9   L is a linker;

10   n is 0 or 1; and

11   Ar' is the residue of a cyclic aliphatic, cyclic heteroaliphatic, aromatic or heteroaromatic

12   moiety optionally substituted with 1-3 noninterfering substituents.

1   7.   The method of claim 6 wherein said compound is a quinazoline derivative.

1   8.   The method of claim 7 wherein Z^3 is N; and Z^5-Z^8 are CR^2.

1   9.   The method of claim 7 wherein Z^3 is N; and at least one of Z^5-Z^8 is nitrogen.

1   10. The method of claim 7 wherein R^3 is an optionally substituted phenyl moiety.

1   11. The method of claim 10 wherein R^3 is selected from the group consisting of 2-, 4-, 5-,

2   2,4- and 2,5-substituted phenyl moieties.

1   12. The method of claim 11 wherein at least one substituent of said phenyl moiety is an

2   alkyl(1-6C), or halo.

1   13. The method of claim 1 wherein said molecule is a compound of formula (2)

2

(2).

3

4   and the pharmaceutically acceptable salts and prodrug forms thereof; wherein

5   Ar represents an optionally substituted aromatic or optionally substituted heteroaromatic

6   moiety containing 5-12 ring members wherein said heteroaromatic moiety contains one or

7   more O, S, and/or N;

8   X is NR^1, O, or S;

9   R^1 is H, alkyl (1-8C), alkenyl (2-8C), or alkynyl (2-8C);

10   Z represents N or CR^4;

11   each of R^3 and R^4 is independently H, or a non-interfering substituent;

12   each R^2 is independently a non-interfering substituent; and

13   n is 0, 1, 2, 3, 4, or 5.

14. A method for reversing the effect of TGF-β-mediated cell activation on the expression of a gene associated with fibrosis, comprising contacting a cell or tissue in which the expression of said gene is altered as a result of TGF-β-mediated cell activation, with a non-peptide small molecule inhibitor of TGF-β, specifically binding a TGFβ-R1 receptor kinase present in said cell or tissue.

15. The method of claim 14 wherein said gene is associated with fibrosis.

16. The method of claim 15 wherein said gene is overexpressed as a result of TGF-β-mediated cell activation.

17. The method of claim 16 wherein said gene is selected from the group consisting of fibronectin, collagen, type I, alpha 2 (COL1A2); collagen, type V, alpha 2 (COL5A2); connective tissue growth factor (CTGF); thrombospondin 1 (THBS1); hexabrachion (HXB); tissue inhibitor of metalloproteinase 1 (TIMP-1); tissue inhibitor of metalloproteinase 3 (TIMP3); plasminogen activator inhibitor-1 (PAI-1); and collagen, type III, alpha 1 (COL3A1).

18. The method of claim 17 wherein said inhibitor reverses the effect of TGF-β-mediated cell activation on the expression of two or more of said genes.

19. The method of claim 18 wherein said gene is underexpressed as a result of TGF-β-mediated cell activation.

20. The method of claim 19 wherein said gene is platelet-derived growth factor receptor-α (PDGFRα).

21. The method of claim 20 wherein said inhibitor reverses the effect of TGF-β-mediated cell activation on the expression of two or more of said genes.

22. The method of claim 21 wherein said tissue is selected from the group consisting of lung tissue, heart tissue, liver tissue, and kidney tissue.

23. The method of claim 22 wherein said inhibitor reverses the effect of TGF-β-mediated cell activation on a multiplicity of genes associated with fibrosis.

24. The method of claim 14 wherein said inhibitor additionally blocks biological activities mediated by Smad proteins, p38 and TAK1.

25. The method of claim 14 wherein said inhibitor is of the formula
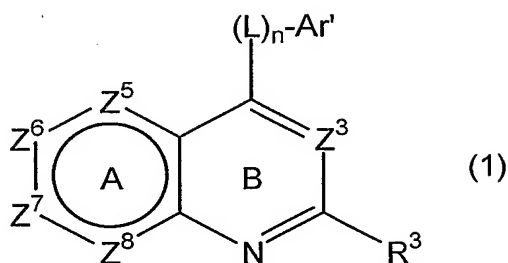
$(L)_n\text{-Ar'}$

(1)

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4   and the pharmaceutically acceptable salts and prodrug forms thereof

5   wherein R^3 is a noninterfering substituent;

6   each Z is CR^2 or N, wherein no more than two Z positions in ring A are N, and wherein

7   two adjacent Z positions in ring A cannot be N;

8   each R^2 is independently a noninterfering substituent;

9   L is a linker;

10   n is 0 or 1; and

11   Ar' is the residue of a cyclic aliphatic, cyclic heteroaliphatic, aromatic or heteroaromatic

12   moiety optionally substituted with 1-3 noninterfering substituents.

1   26. The method of claim 25 wherein said compound is a quinazoline derivative.

1   27. The method of claim 26 wherein Z^3 is N; and $Z^5\text{-}Z^8$ are CR^2.

1   28. The method of claim 27 wherein Z^3 is N; and at least one of $Z^5\text{-}Z^8$ is nitrogen.

1   29. The method of claim 27 wherein R^3 is an optionally substituted phenyl moiety.

1   30. The method of claim 29 wherein R^3 is selected from the group consisting of 2-, 4-

2   , 5-, 2,4- and 2,5-substituted phenyl moieties.

1   31. The method of claim 30 wherein at least one substituent of said phenyl moiety is

2   an alkyl(1-6C), or halo.

1   32. The method of claim 14 wherein said molecule is a compound of formula (2)
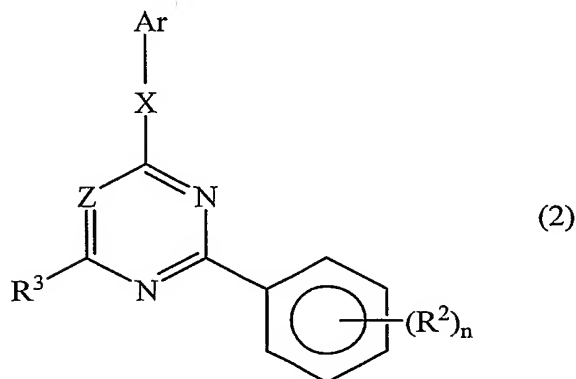
2

(2)

3

4   and the pharmaceutically acceptable salts and prodrug forms thereof; wherein

5   Ar represents an optionally substituted aromatic or optionally substituted heteroaromatic

6 moiety containing 5-12 ring members wherein said heteroaromatic moiety contains one or

7 more O, S, and/or N;

8   X is NR^1, O, or S;

9   R^1 is H, alkyl (1-8C), alkenyl (2-8C), or alkynyl (2-8C);

10   Z represents N or CR^4;

11   each of R^3 and R^4 is independently H, or a non-interfering substituent;

12   each R^2 is independently a non-interfering substituent; and

13   n is 0, 1, 2, 3, 4, or 5.

1   33. A method for determining the likelihood of a positive response of a subject

2 diagnosed with a fibroproliferative disease associated with TGF-β-mediated cell activation to

3 treatment with a TGF-β inhibitor specifically binding the TGFβ-R1 receptor, comprising

4   (a)   determining in a biological sample obtained from said subject the expression level

5 of one or more genes selected from the group consisting of fibronectin (FN), collagen, type I,

6 alpha 2 (COL1A2); collagen, type V, alpha 2 (COL5A2); connective tissue growth factor

7 (CTGF); thrombospondin 1 (THBS1); hexabrachion (HXB); tissue inhibitor of

8 metalloproteinase 1 (TIMP-1); tissue inhibitor of metalloproteinase 3 (TIMP3); plasminogen

9 activator inhibitor-1 (PAI-1); platelet-derived growth factor receptor-α (PDGFRα);

10 glucocorticoid receptor (GR); Smad2; Smad3; Smad4; Smad7; Col 1; Col 3; TGF-β activated

11 kinase (TAKI); p38 alpha; β-actin; Cox1; Cox 2; I kappa-B kinase (iKKi); and collagen, type III,

12 alpha-1 (COL3A1), compared with expression in a sample obtained from a normal subject; and

13   (b)   indicating a positive response, if one or more of said genes are differentially

14 expressed.

1   34. The method of claim 33 wherein a positive response is indicated if one or more

2 genes selected from the group consisting of fibronectin (FN), collagen, type I, alpha 2

3 (COL1A2); collagen, type V, alpha 2 (COL5A2); connective tissue growth factor (CTGF);

4 thrombospondin 1 (THBS1); hexabrachion (HXB); tissue inhibitor of metalloproteinase 1

5 (TIMP-1); tissue inhibitor of metalloproteinase 3 (TIMP3); plasminogen activator inhibitor-1

6 (PAI-1); Smad7; Col 1; interleukin-6 (IL-6); Cox1; Cox2; and collagen, type III, alpha 1

7 (COL3A1) are overexpressed.

1   35. The method of claim 33 wherein a positive response is indicated if one or more of

2 genes selected from the group consisting of platelet-derived growth factor receptor-α

3 (PDGFRα); glucocorticoid receptor (GR); Smad3; and I kappa-B kinase (iKKi) are

4 underexpressed.

36. The method of claim 33 wherein said fibroproliferative disease is selected from the group consisting of glomerulonephritis (GN); diabetic nephropathy; renal interstitial fibrosis; renal fibrosis resulting from complications of drug exposure; HIV-associated nephropathy; transplant necropathy; liver cirrhosis due to all etiologies; disorders of the biliary tree; hepatic dysfunction attributable to infections; pulmonary fibrosis; adult respiratory distress syndrome (ARDS); chronic obstructive pulmonary disease (COPD); idiopathic pulmonary fibrosis (IPF); acute lung injury (ALI); congestive heart failure; dilated cardiomyopathy; myocarditis; vascular stenosis; progressive systemic sclerosis; polymyositis; scleroderma; dermatomyositis; fascists; Raynaud's syndrome, rheumatoid arthritis; proliferative vitreoretinopathy; fibrosis associated with ocular surgery; and excessive or hypertrophic scar or keloid formation in the dermis occurring during wound healing resulting from trauma or surgical wounds.

37. A method of diagnosing a patient with a fibroproliferative disease, comprising

    (a)   determining in a biological sample obtained from said patient the expression level of one or more genes selected from the group consisting of fibronectin, collagen, type I, alpha 2 (COL1A2); collagen, type V, alpha 2 (COL5A2); connective tissue growth factor (CTGF); thrombospondin 1 (THBS1); hexabrachion (HXB); tissue inhibitor of metalloproteinase 1 (TIMP-1); tissue inhibitor of metalloproteinase 3 (TIMP3); plasminogen activator inhibitor-1 (PAI-1); collagen, type III, alpha 1 (COL3A1); glucocorticoid receptor (GR); Smad2; Smad3; Smad4; Smad7; Col 1; Col 3; TGF-β activated kinase (TAKI); p38 alpha; β-actin; Cox1; Cox 2; I kappa-B kinase (iKKi); and platelet-derived growth factor receptor-α (PDGFRα), compared with expression in a normal sample; and

    (b)   diagnosing said patient with a fibroproliferative disease if one or more of said genes are differentially expressed.

38. The method of claim 37 wherein said patient is diagnosed with said fibroproliferative disease if one or more genes selected from the group consisting of fibronectin (FN), collagen, type I, alpha 2 (COL1A2); collagen, type V, alpha 2 (COL5A2); connective tissue growth factor (CTGF); thrombospondin 1 (THBS1); hexabrachion (HXB); tissue inhibitor of metalloproteinase 1 (TIMP-1); tissue inhibitor of metalloproteinase 3 (TIMP3); plasminogen activator inhibitor-1 (PAI-1); Smad7; Col 1; interleukin-6 (IL-6); Cox1; Cox2; and collagen, type III, alpha-1 are overexpressed.

39. The method of claim 37 wherein said patient is diagnosed with a fibroproliferative disease if one or more of genes selected from the group consisting of platelet-derived growth factor receptor-α (PDGFRα); glucocorticoid receptor (GR); Smad3; and I kappa-B kinase (iKKi) are underexpressed.

1   40. A method of treating a fibroproliferative disease in a subject, comprising

2   administering to the subject dexamethasone and a TGF-β inhibitor, specifically binding a

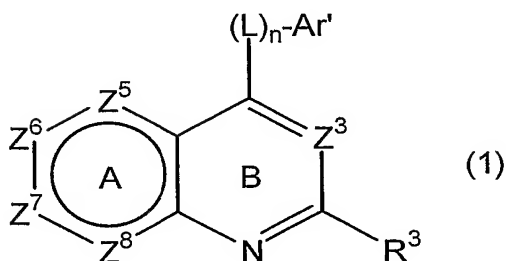3   TGFβ-R1 receptor.

1   41. The method of claim 40 wherein the fibroproliferative disease is a renal, hepatic,

2   pulmonary, cardiovascular, eye, or dermatological disorder associated with enhanced TGF-β

3   activity and excessive fibrosis or sclerosis.

1   42. The method of claim 40 wherein said fibroproliferative disease is selected from

2   the group consisting of glomerulonephritis (GN); diabetic nephropathy; renal interstitial

3   fibrosis; renal fibrosis resulting from complications of drug exposure; HIV-associated

4   nephropathy; transplant necropathy; liver cirrhosis due to all etiologies; disorders of the

5   biliary tree; hepatic dysfunction attributable to infections; pulmonary fibrosis; adult

6   respiratory distress syndrome (ARDS); chronic obstructive pulmonary disease (COPD);

7   idiopathic pulmonary fibrosis (IPF); acute lung injury (ALI); pulmonary fibrosis due to

8   infectious or toxic agents; congestive heart failure; dilated cardiomyopathy; myocarditis;

9   vascular stenosis; progressive systemic sclerosis; polymyositis; scleroderma;

10 dermatomyositis; fascists; Raynaud's syndrome, rheumatoid arthritis; proliferative

11 vitreoretinopathy; fibrosis associated with ocular surgery; and excessive or hypertrophic scar

12 or keloid formation in the dermis occurring during wound healing resulting from trauma or

13 surgical wounds.

1   43. The method of claim 40 wherein the subject is human.

1   44. The method of claim 43 wherein said TGF-β inhibitor is a compound of formula

2   (1)

3

4   and the pharmaceutically acceptable salts and prodrug forms thereof

5   wherein R^3 is a noninterfering substituent;

6   each Z is CR^2 or N, wherein no more than two Z positions in ring A are N, and

7   wherein two adjacent Z positions in ring A cannot be N;

8   each R^2 is independently a noninterfering substituent;

9   L is a linker;

11   n is 0 or 1; and

12   Ar' is the residue of a cyclic aliphatic, cyclic heteroaliphatic, aromatic or heteroaromatic

13   moiety optionally substituted with 1-3 noninterfering substituents.

1   45. The method of claim 43 wherein said TGF-β inhibitor if a compound of formula

2   (2)

3

(2)

4

5   and the pharmaceutically acceptable salts and prodrug forms thereof; wherein

6   Ar represents an optionally substituted aromatic or optionally substituted heteroaromatic

7   moiety containing 5-12 ring members wherein said heteroaromatic moiety contains one or

8   more O, S, and/or N;

9   X is NR^1, O, or S;

10   R^1 is H, alkyl (1-8C), alkenyl (2-8C), or alkynyl (2-8C);

11   Z represents N or CR^4;

12   each of R^3 and R^4 is independently H, or a non-interfering substituent;

13   each R^2 is independently a non-interfering substituent; and

14   n is 0, 1, 2, 3, 4, or 5.

FIGURE 1

FIGURE 2

Inhibition effect of Test Compound on PAI-1 secretion from HLF stimulated with TGFb at 48 hours.
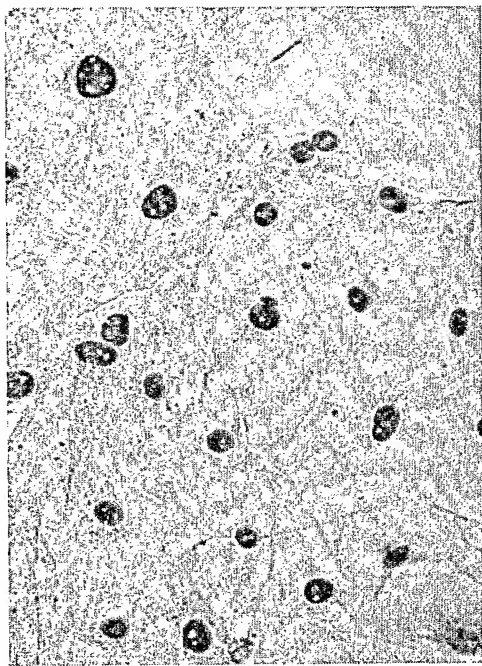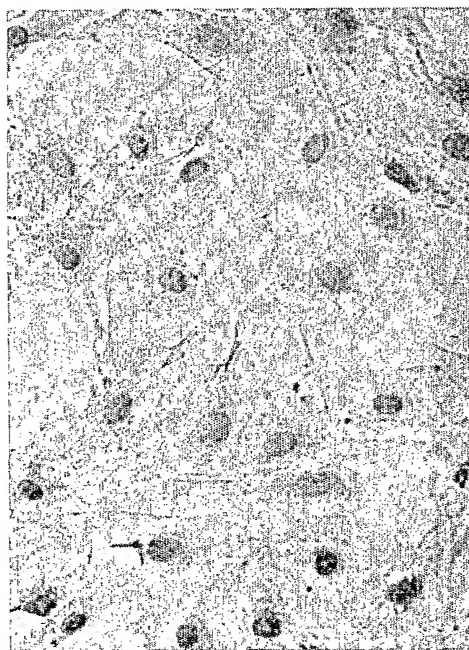
FIGURE 3

Effect of Test Compound on CTGF intracellular protein expression from RLF in the time course of 48 hours
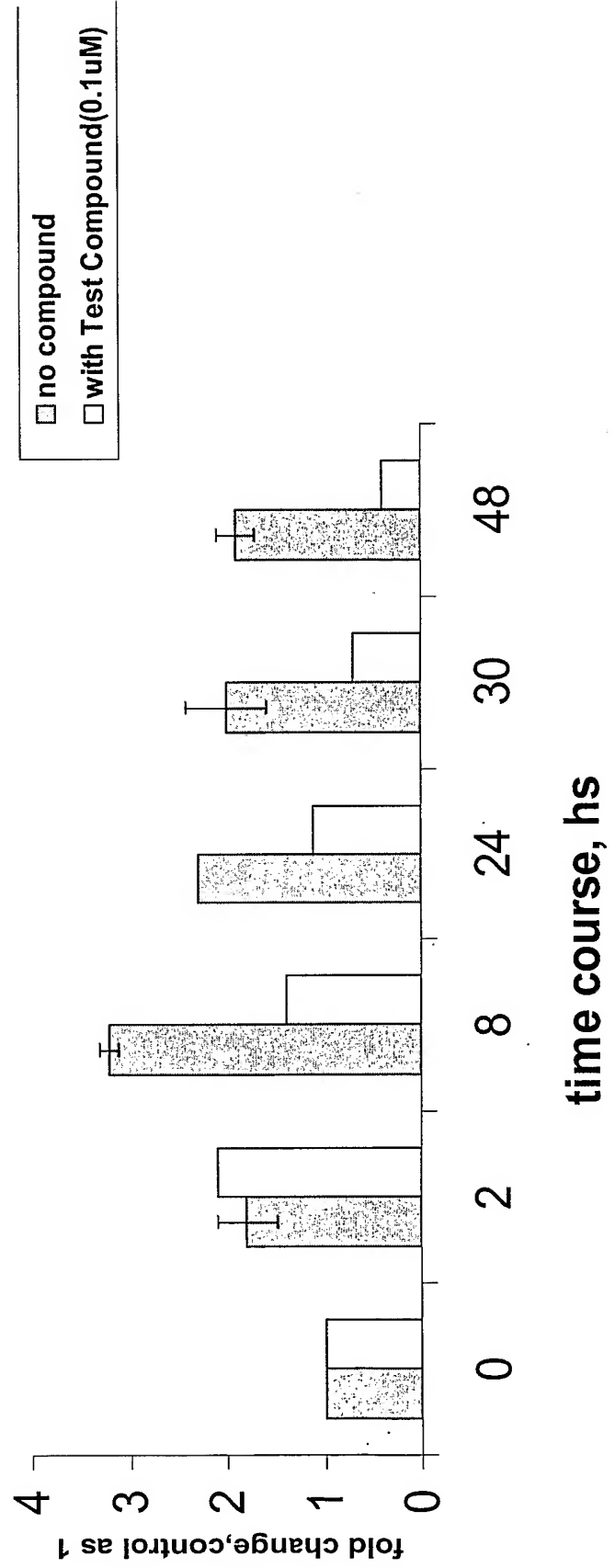
FIGURE 4

Inhibition of TGFb-Induced PAI-1 Protein by Test Compounds in Hep G2 Cells

Compound	EC$_{50}$, uM
Test Compound 5	0.01
Test Compound 5	0.01
Test Compound 2	0.04
Test Compound 3	0.025

PAI-1 Assay, 5 x 10^3 Hep G2 Cells, 10 ng/ml TGF-B + Inhibitors
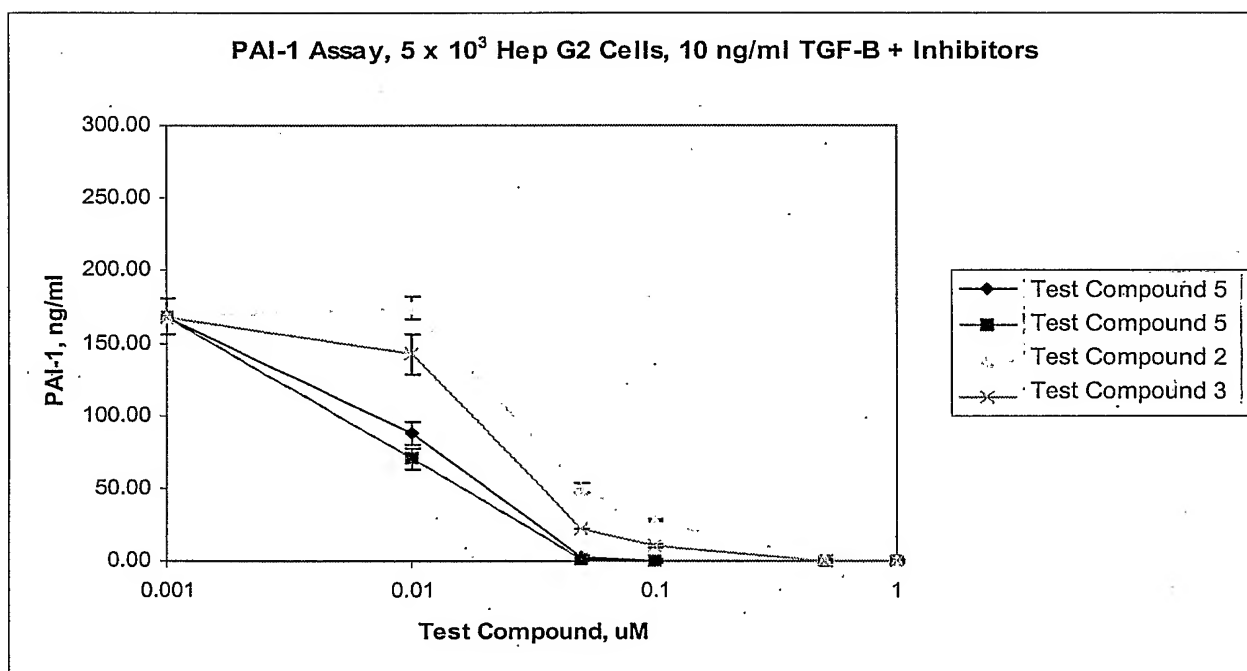
FIGURE 5

TGFβ Stimulated Fibrotic Genes Affected by Test Compound 6 (TC 6) and Test Compound 5 (TC 5)

mcid	RLF TGFb 24hr *in vitro*			NRK TGFb 24hr *in vitro*				Best Name	Best Accession
	TGFb	TGFb TC6	TGFb TC5	TGFb	TGFb TC6	TGFb TC5			
P01033_H08	1.2	-1.1	-1.5	1.9	-1.5	-2.6	COL1A2	collagen, type I, alpha 2	NM_000089
P00637_E06	-1.2	1.3	1.3	-3.0	-2.5	-5.1	COL3A1	collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV,	NM_000090
P00537_A12	-1.2	1.2	1.2	3.0	-2.5	-4.5	COL3A1	collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV,	NM_000090
P01029_F09	-1.3	1.1	1.2	3.4	-2.5	-4.6	COL3A1	collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV,	NM_000090
P00871_D04	-1.1	-1.8	1.4	1.9	-1.2	-1.5	COL4A1	collagen, type IV, alpha 1	NM_001845
P00822_F01	1.3	-1.3	-1.7	3.0	-2.5	-3.8	COL5A2	collagen, type V, alpha 2	NM_000393
P01044_B02	2.8	-1.7	-27.3	-1.1	-1.1	-1.1	CTGF	connective tissue growth factor	NM_001901
P00537_A09	1.1	1.1	1.2	2.0	-1.8	-2.0	FN1	fibronectin 1	NM_019143.1
P01056_C09	2.8	-2.0	-5.5	2.3	-2.8	-3.1	HXB	hexabrachion (tenascin C, cytotactin)	NM_002160
P01029_D04	-2.3	1.6	5.0	-2.0	1.7	1.7	PDGFRA	platelet-derived growth factor receptor, alpha polypeptide	NM_006206
P00637_A10	8.9	-3.7	-35.8	1.3	-2.4	-2.0	SERPINE	serine (or cysteine) proteinase inhibitor, clade E (nexin,	NM_000602
P01030_G08	9.8	-4.1	-43.5	1.4	-2.5	-2.0	SERPINE	serine (or cysteine) proteinase inhibitor, clade E (nexin,	NM_000602
P00771_A07	3.7	-3.0	-17.5	-1.1	1.0	-1.2	THBS1	thrombospondin 1	NM_003246
P00825_B07	2.3	-1.9	-3.4	1.5	-1.5	-1.7	TIMP1	tissue inhibitor of metalloproteinase 1 (erythroid potentiating	NM_003254
P01048_G06	1.0	-1.2	-1.4	4.0	-3.3	-3.8	TIMP3	tissue inhibitor of metalloproteinase 3 (Sorsby fundus	NM_000362

FIGURE 6

TGFβ induced gene expression of osteopontin is reversed by TGFβ-RI Test Compound 6 (TC 6) in Rat Whole Blood Cells at 4 hours
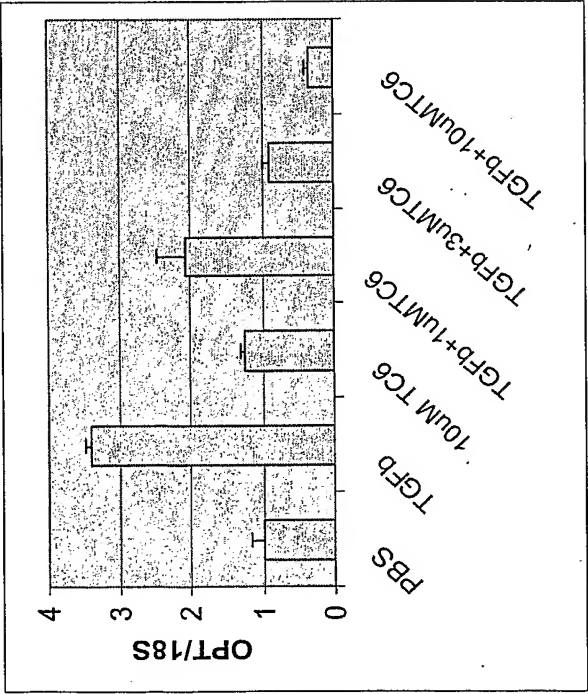
Microarray

MCID	untreated vs TGFb	TGFb vs TGFb_TC6	Description
P00774_E01	-4.13	-2.35	"Rat osteopontin mRNA, complete cds."

Real Time PCR

FIGURE 7

Plasma Concentrations of Test Compound
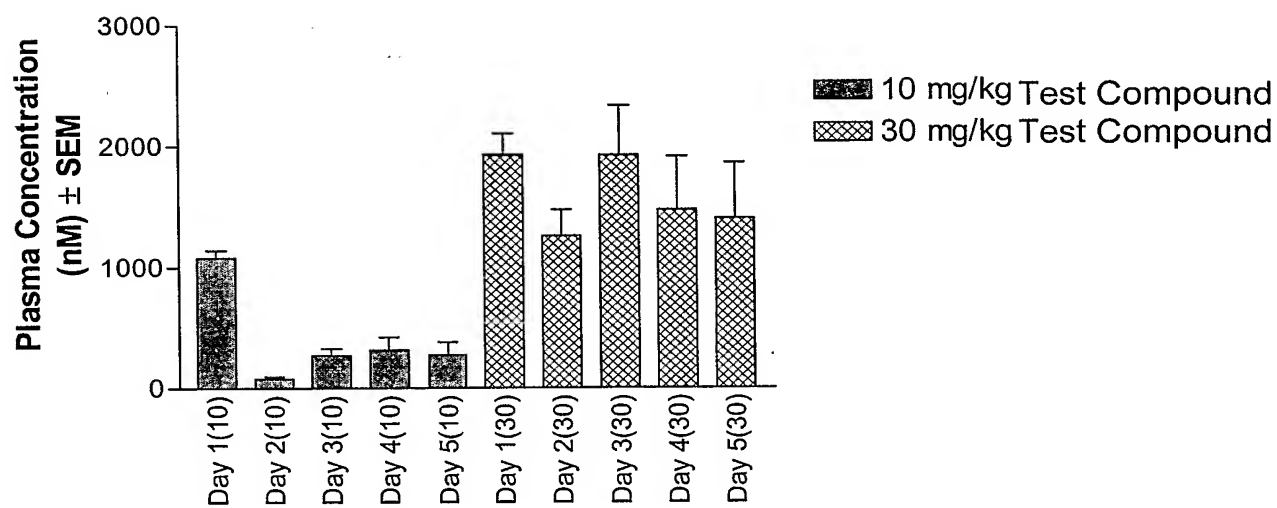
FIGURE 8

Per Cent Body Weight Change From Day 0
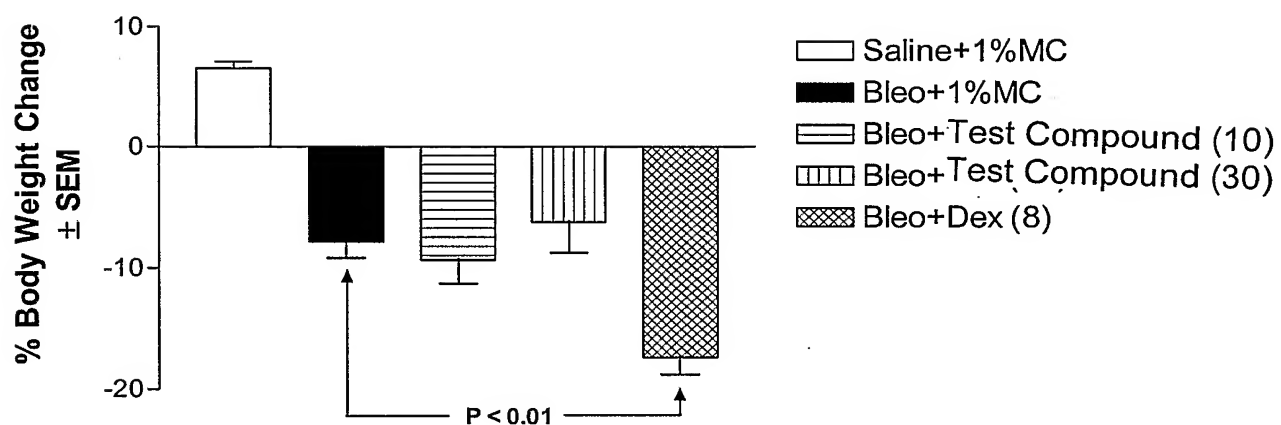
FIGURE 9

Total IL-6 in the BALF
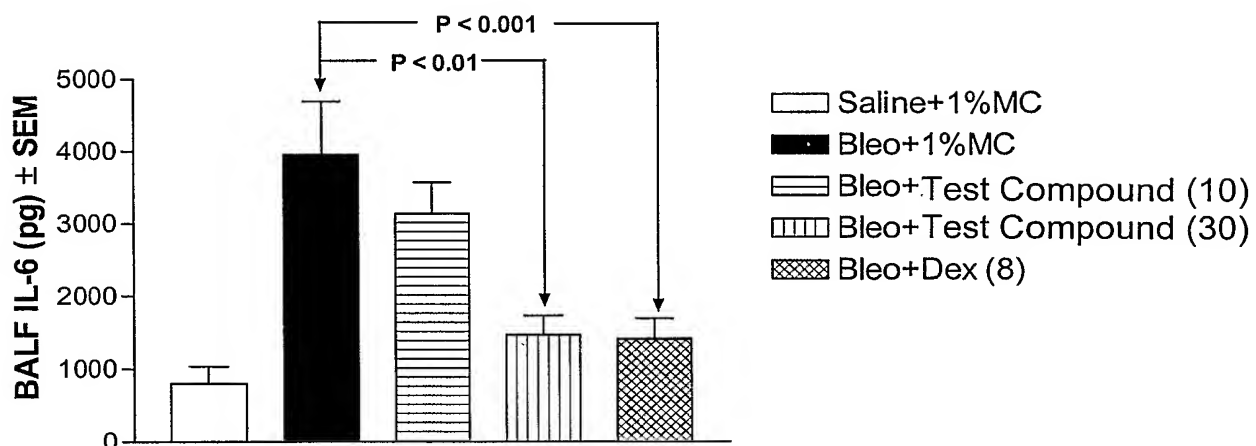
FIGURE 10

PAI-1 mRNA Expression in the Lung Tissues

Legend:
- ☐ Saline+1% MC
- ■ Bleo+1% MC
- ☰ Bleo+Test Compound (10)
- ▦ Bleo+Test Compound (30)
- ▨ Bleo+Dex (8)

P = 0.07

Y-axis: PAI-1 mRNA/18S rRNA ± SEM
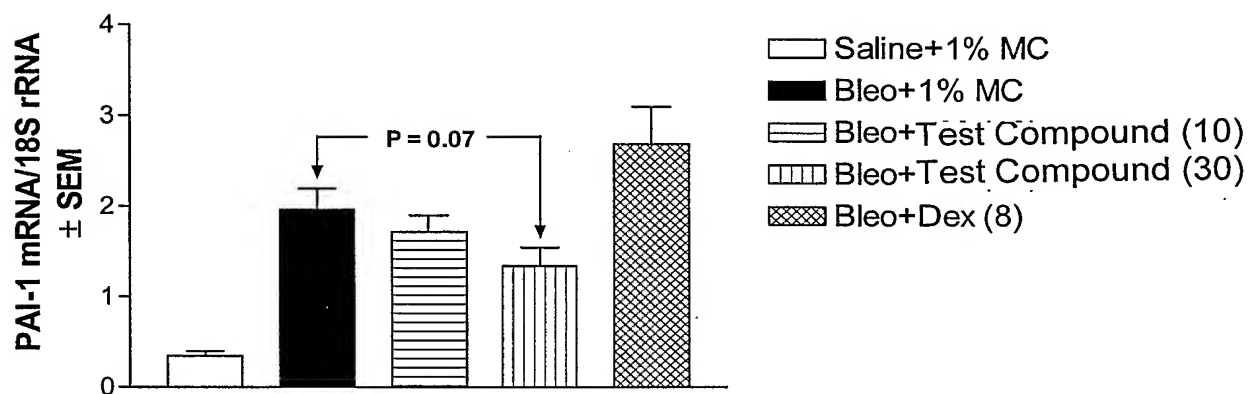
FIGURE 11

CTGF mRNA Expression in the Lung Tissues

$P < 0.01$

$P < 0.02$

- Saline+1% MC
- Bleo+1% MC
- Bleo+Test Compound (10)
- Bleo+Test Compound (30)
- Bleo+Dex (8)
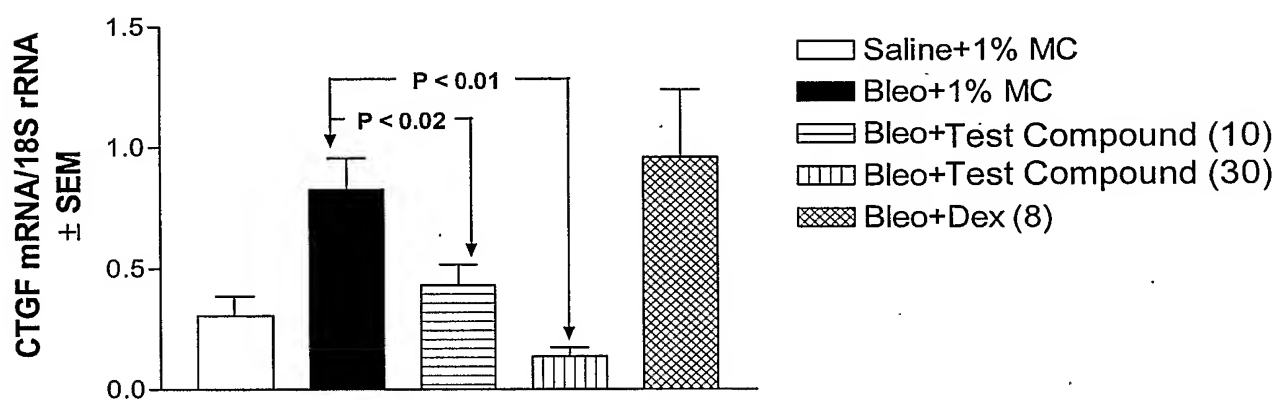
FIGURE 12

TIMP-1 mRNA Expression in the Lung Tissues
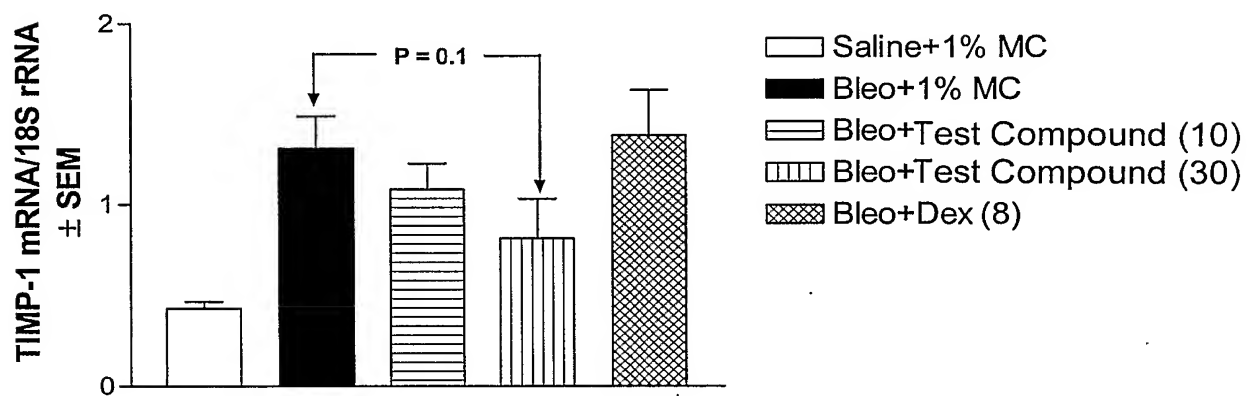
FIGURE 13

Fibronectin mRNA Expression in the Lung Tissues

Legend:
- Saline+1% MC
- Bleo+1% MC
- Bleo+Test Compound (10)
- Bleo+Test Compound (30)
- Bleo+Dex (8)
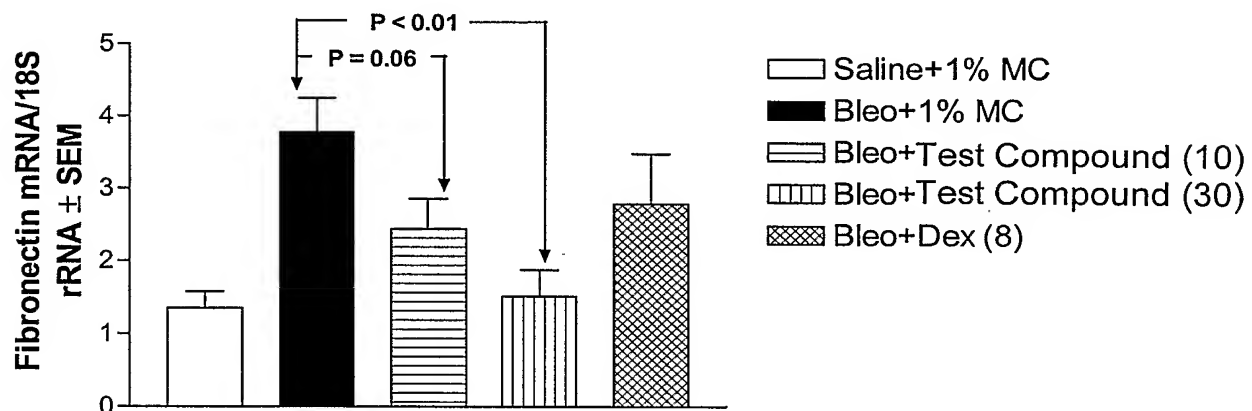
FIGURE 14

Inhibition of α-SMA Protein Expression by Test Compound (TC)

TGFβ1	-	+	-	+
Test Compound	-	-	+	+

a-SMA Protein Expression
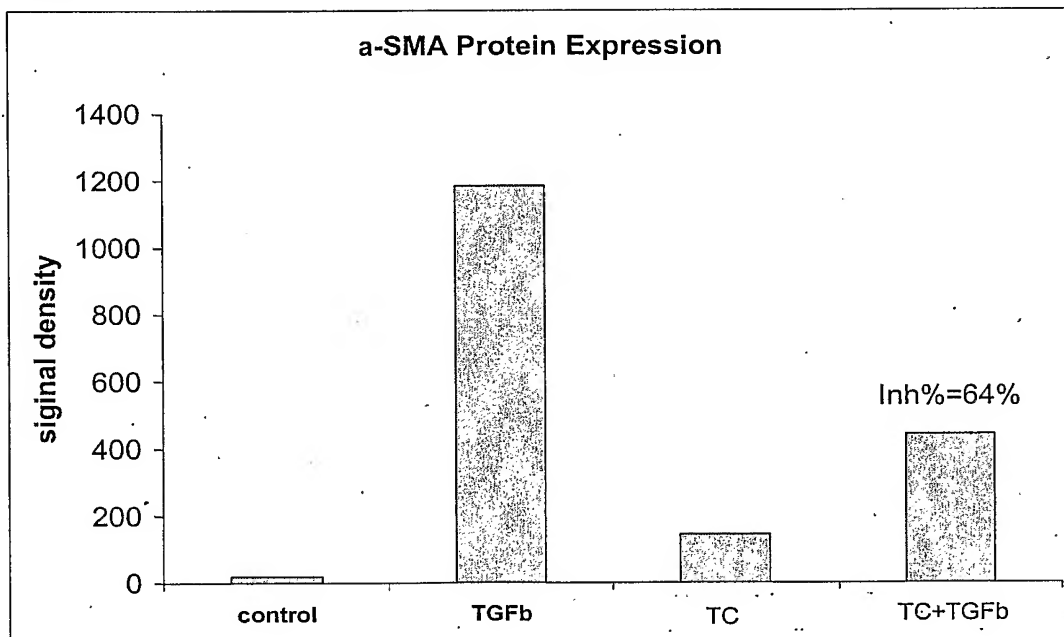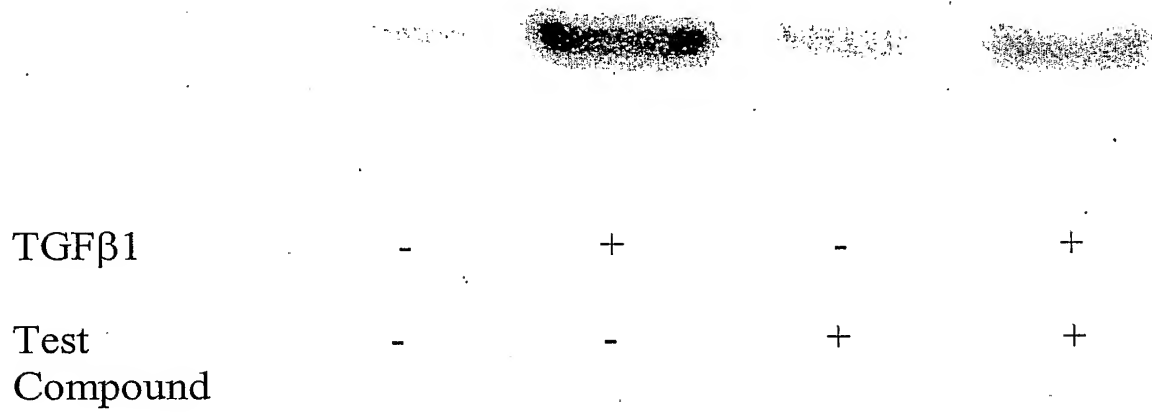
FIGURE 15

Inhibition of IL-6 Protein Expression by Test Compound

IL-6 Protein Secretion, 24 hours
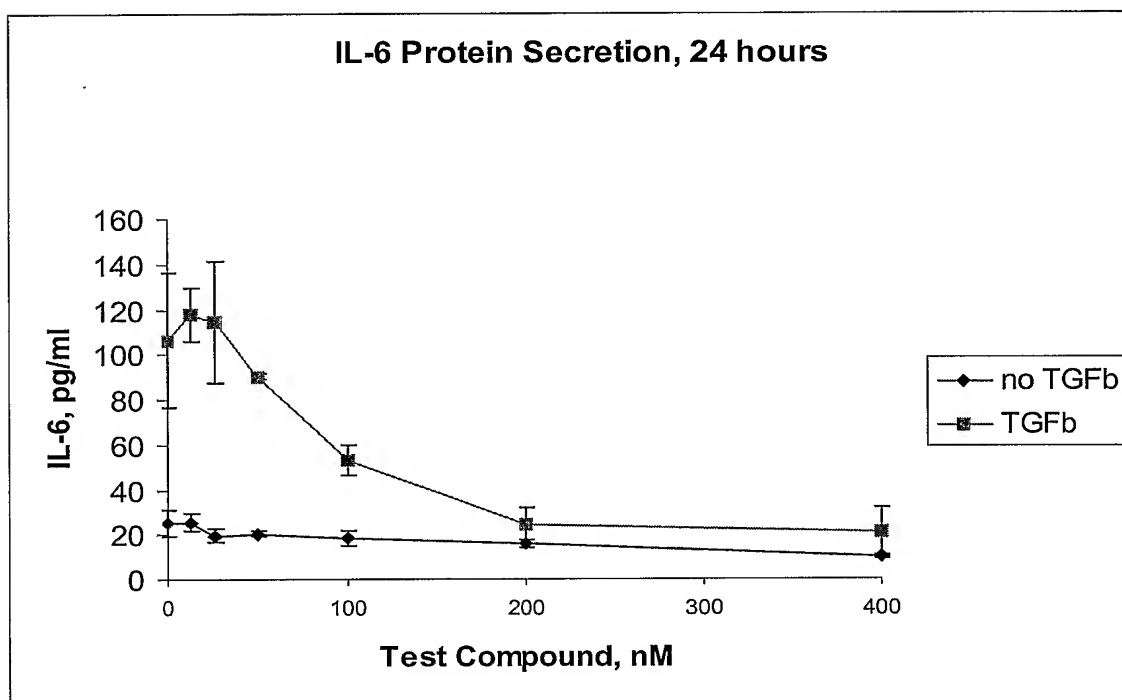
FIGURE 16

Inhibition of PAI-1 Protein Expression by Test Compound
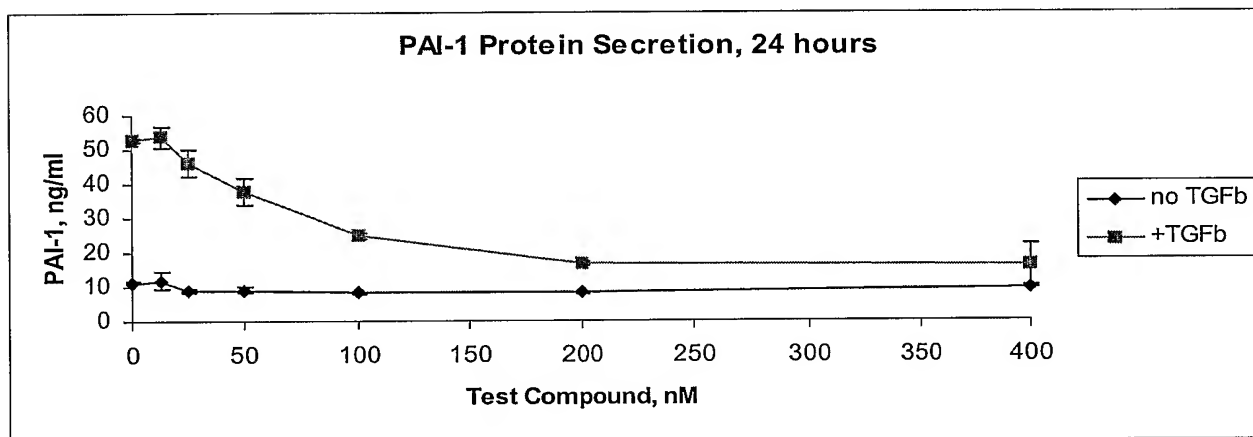
FIGURE 17

Inhibition of Pro-Col 1 C- Peptide Expression by Test Compound

Pro-Col 1 C-Peptide Secretion, 24 hours, EC50=400+/-135nM
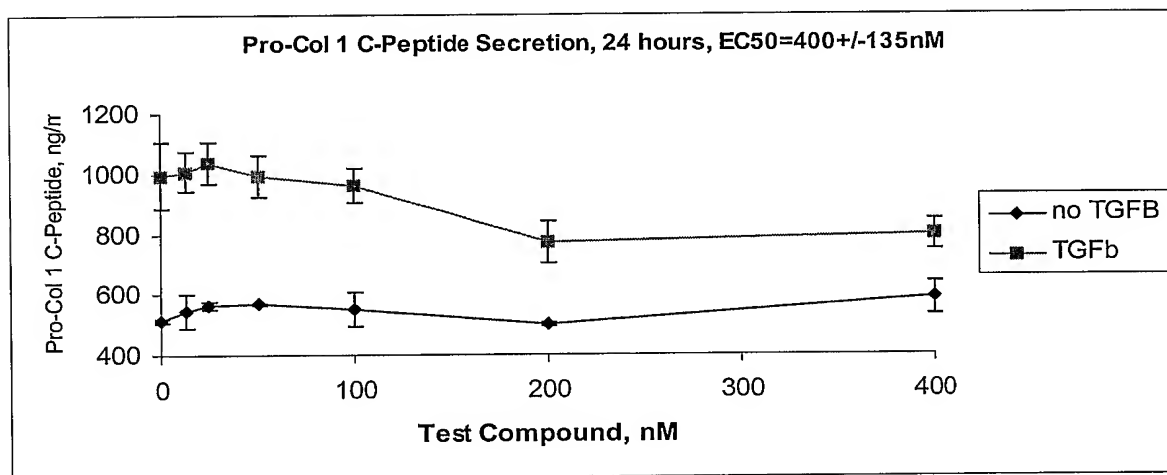
FIGURE 18

Test Compound Blocks TGFβ induced Smad2 phosphorylation in HLF cells

| Test Compound (nM) | 0 | 0 | 25 | 50 | 100 | 150 | 300 |
| TGFβ (2ng/ml) | - | + | + | + | + | + | + |

← P- Smad2

← Smad2

55K →

Effect on P-Smad by Test Compund in HLF cells

Immunoreactivity (AU)

0 / 500 / 1000 / 1500 / 2000

control / 25 nM / 50 nM / 100 nM / 150 nM / 300 nM
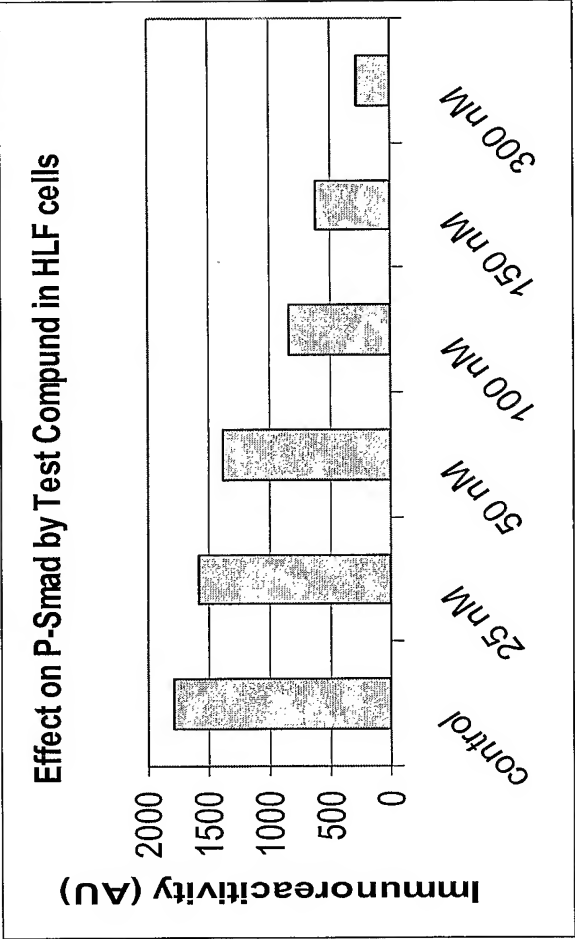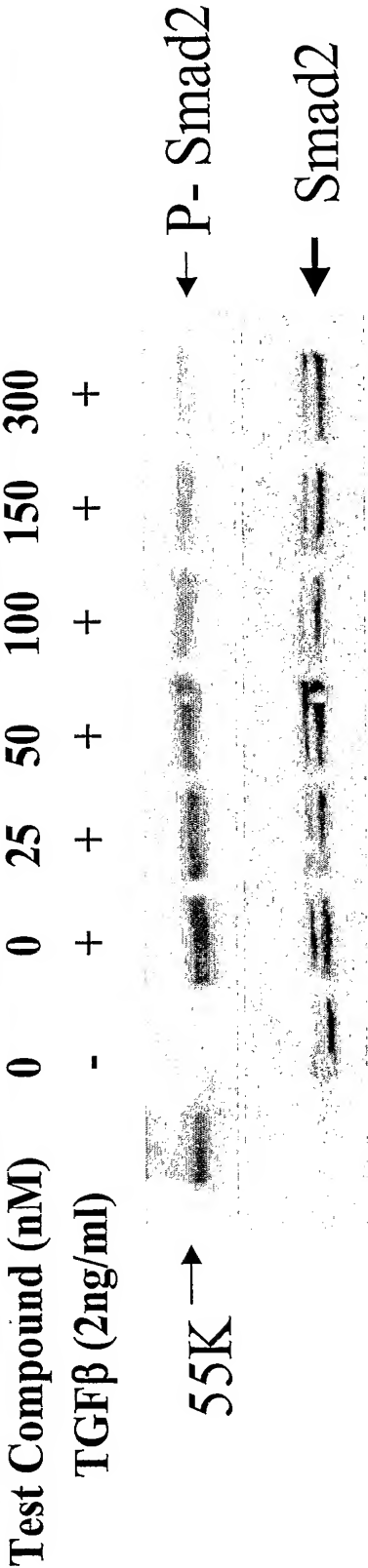
FIGURE 19

Test Compound (100nM) inhibits TGFb induced Smad2 nuclear translocation in HLF cells
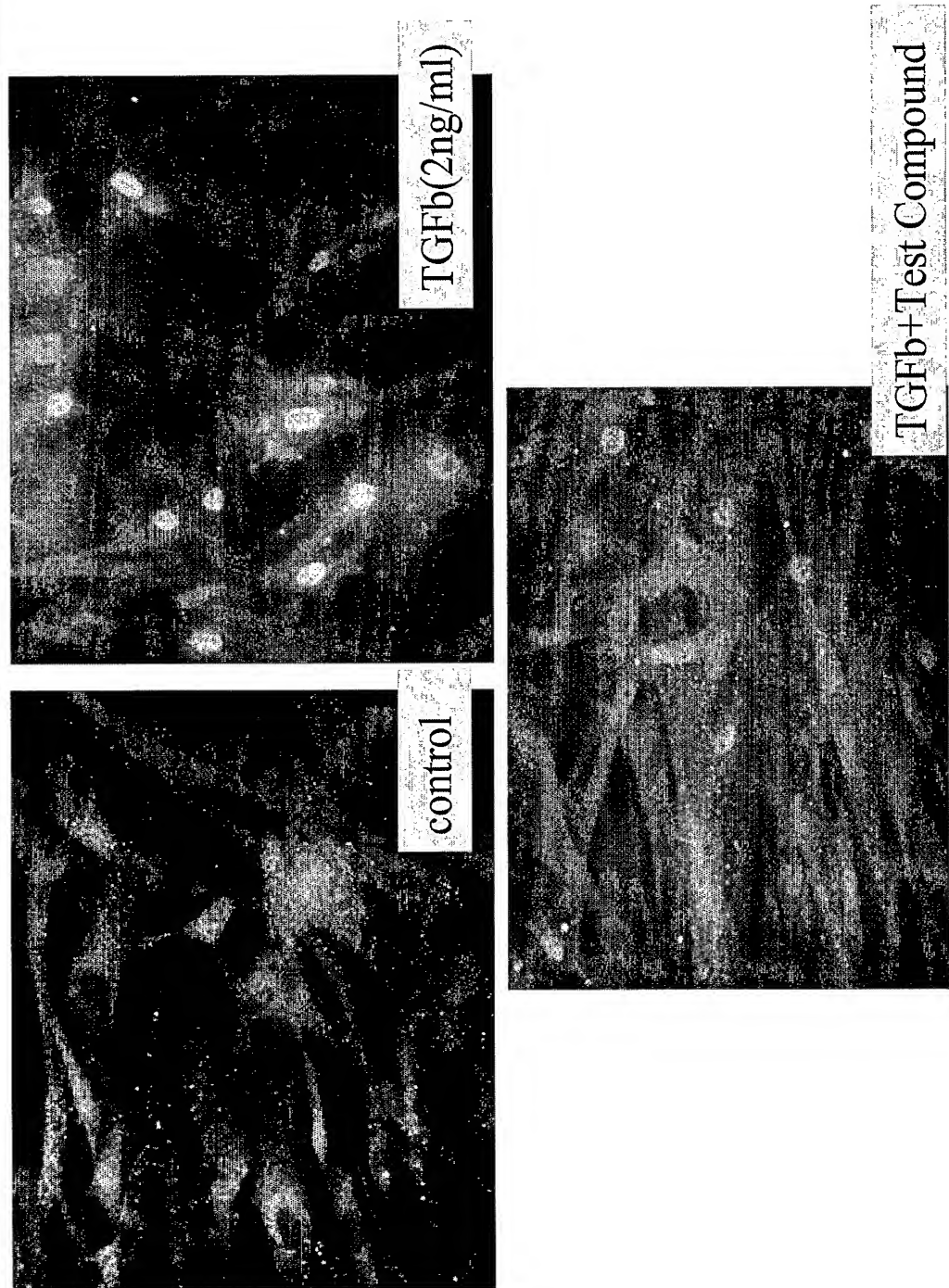
FIGURE 20

Test Compound (100nM) inhibits TGFb induced Smad3 nuclear translocation in HLF cells
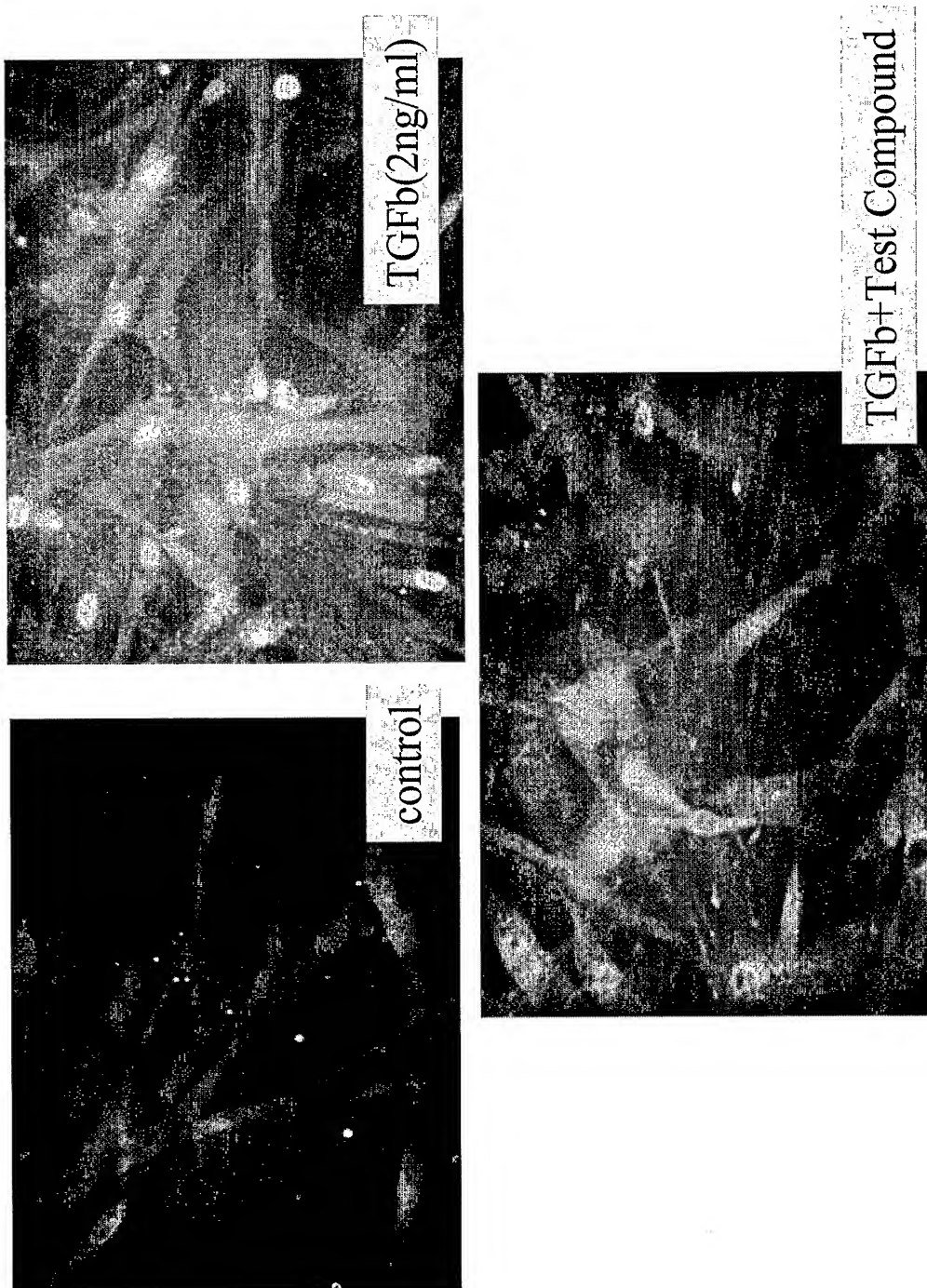
FIGURE 21

Inhibition of Kinases by Test Compound

Kinase Target	IC50 (uM)	% Inhibition at 50uM
TGFb RI Kinase	0.0485	
p38 alpha	0.854	
p38 beta	1.92	
EGF Receptor Kinase	0.686	
p38 gamma		0
JNK1		0
TGFb RII Kinase		22
MAPKAP K2		29
MKK6		37
ERK2		20
PKA		15
PKC		0
PKD		68
cdc2		61
CaMKII		10

FIGURE 22

Inhibition of Activin-induced Hemaglobin Production in K562
Cells by Test Compounds

Hb Assay, K-562 Cells, 10 ng/ml Activin A + Test Compounds

FIGURE 23

FIGURE 24

PAI-1 Expression in HLF cells
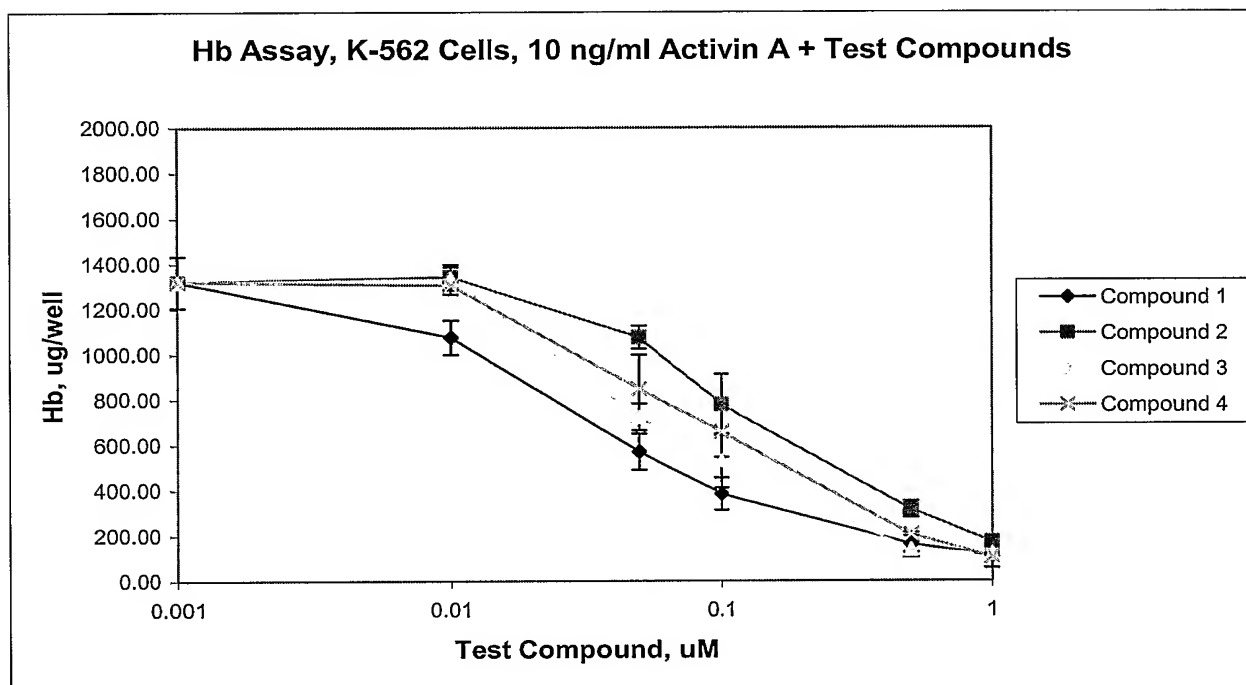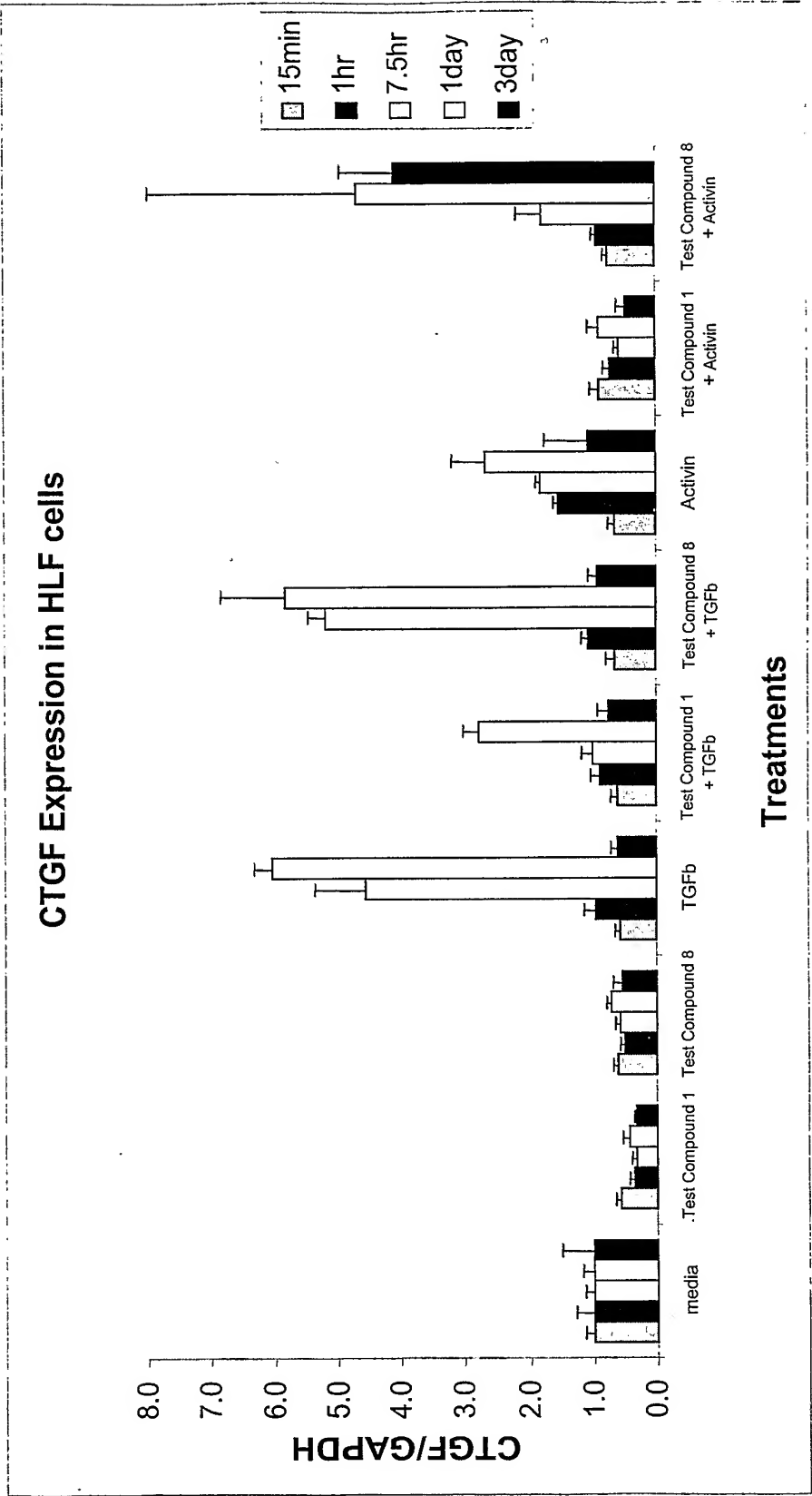
FIGURE 25
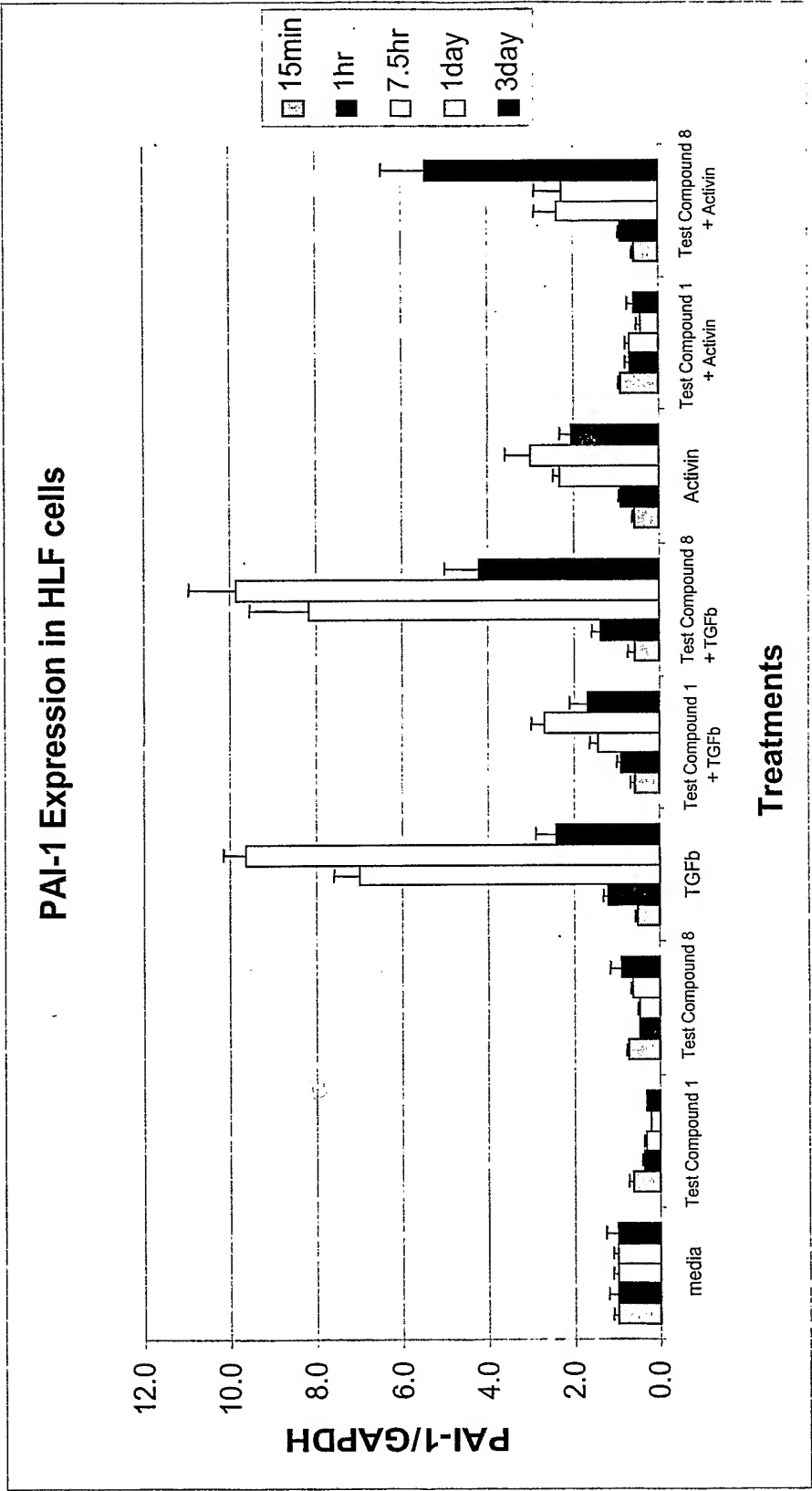
CTGF Expression in HLF (mRNA)
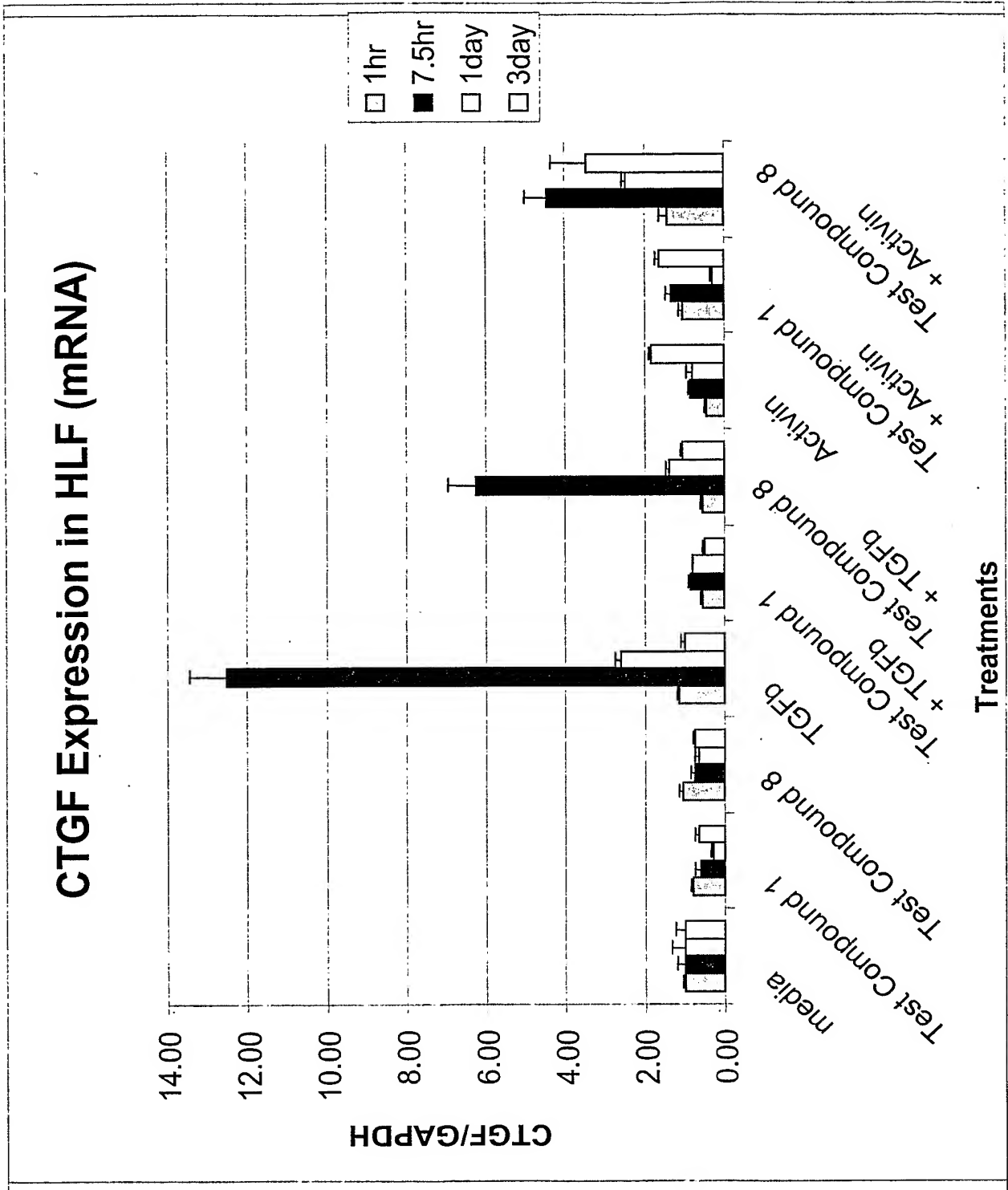
FIGURE 26

PAI-1 Expression in HLF (mRNA)
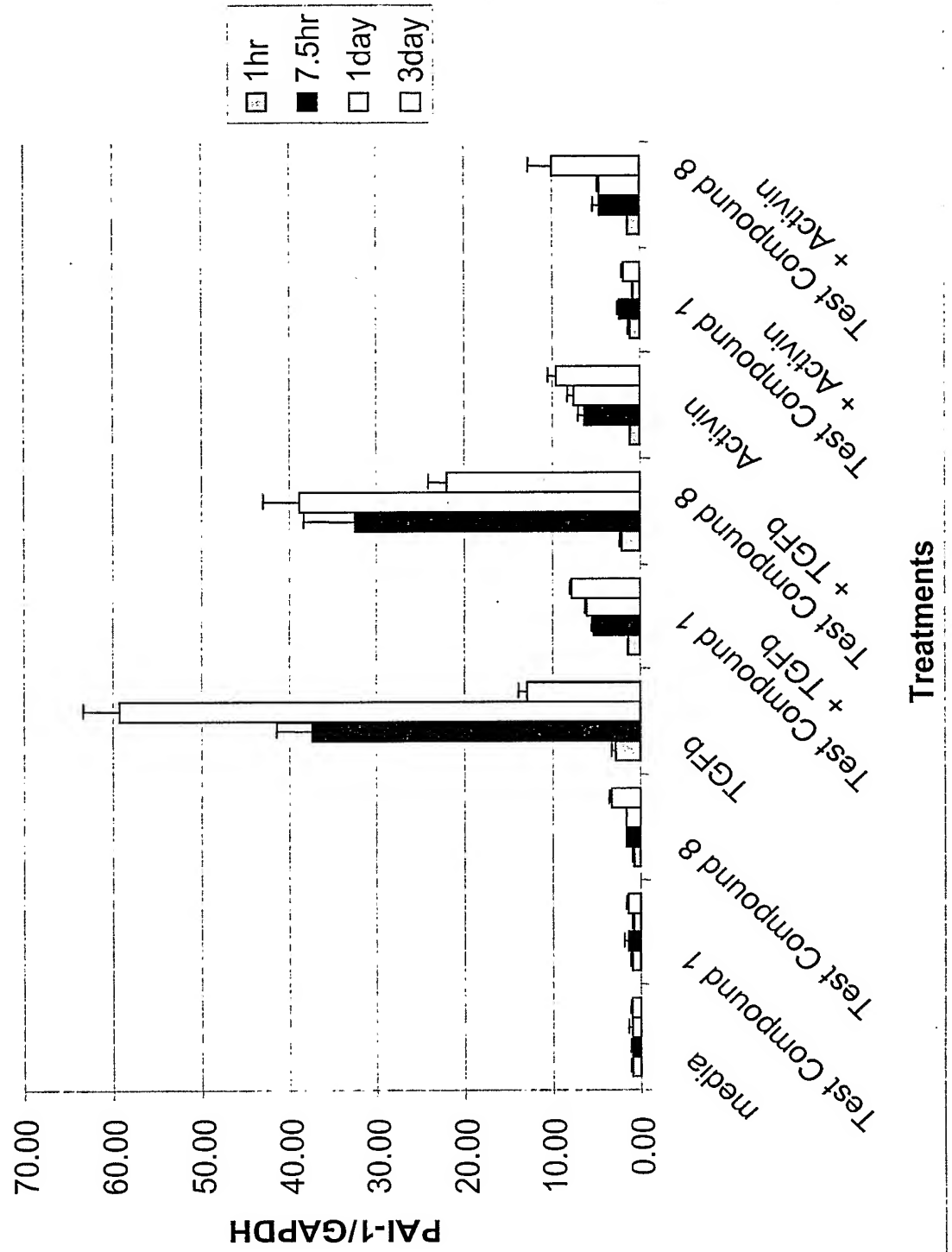
FIGURE 27

A.
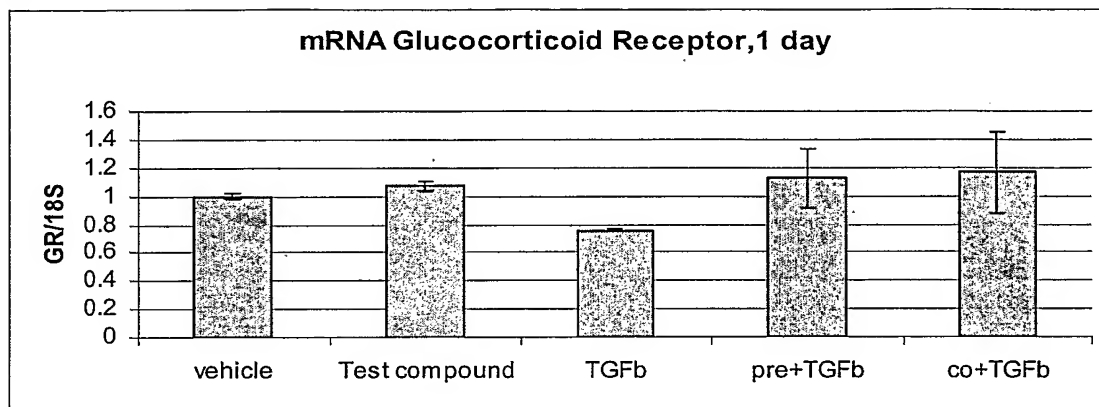
B.
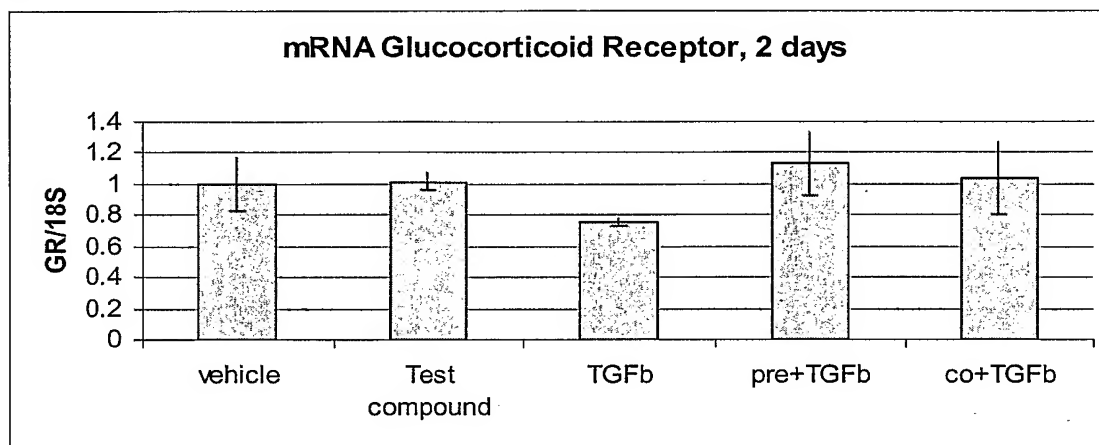
C.
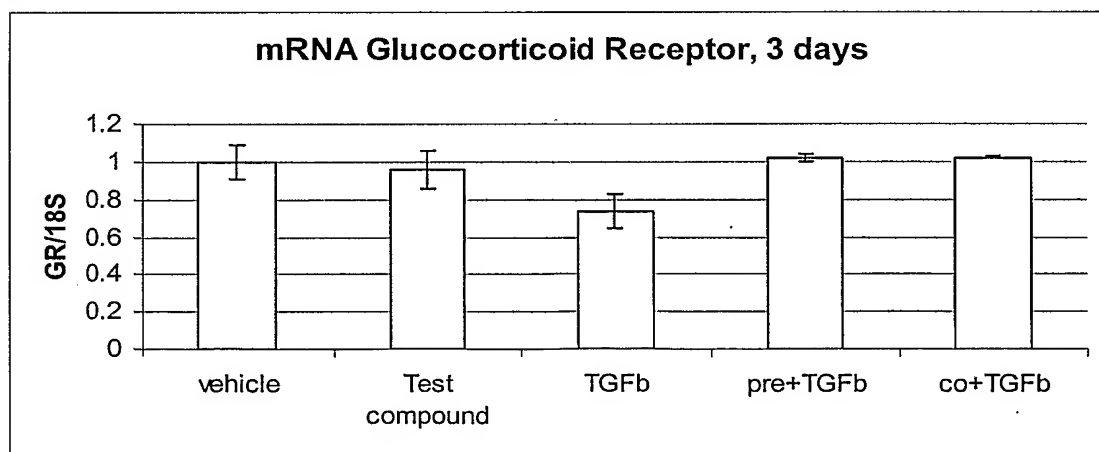
FIGURE 28
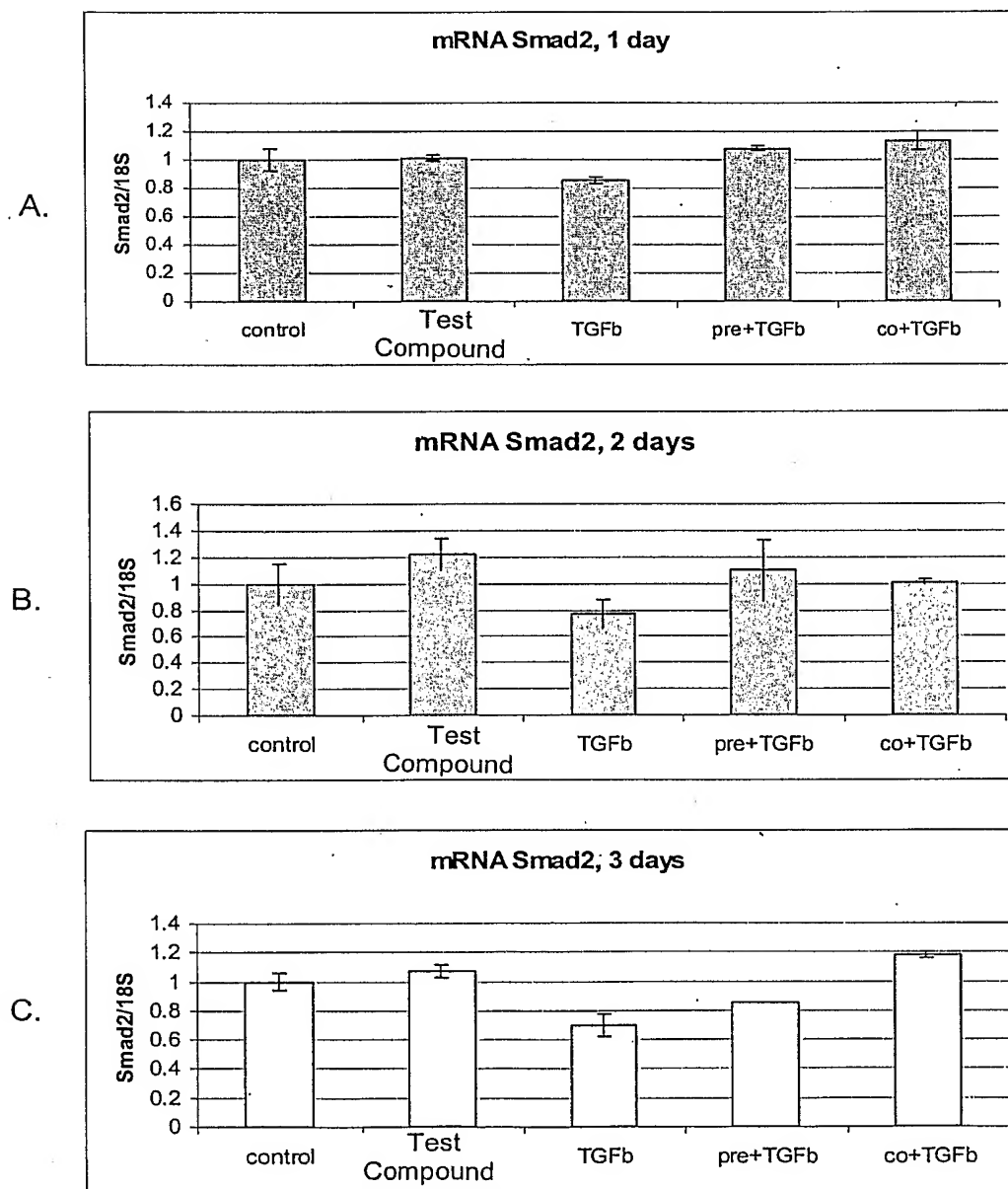
FIGURE 29
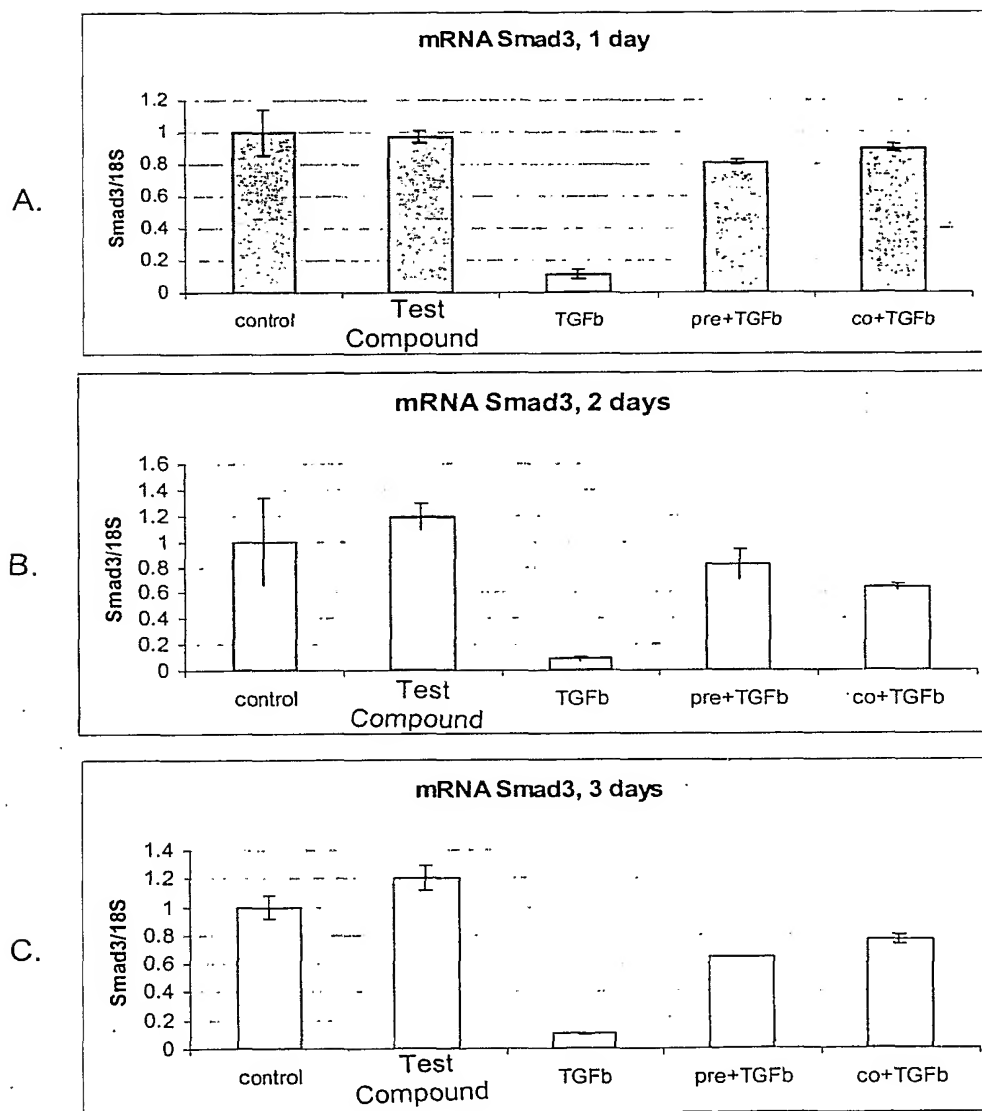
FIGURE 30
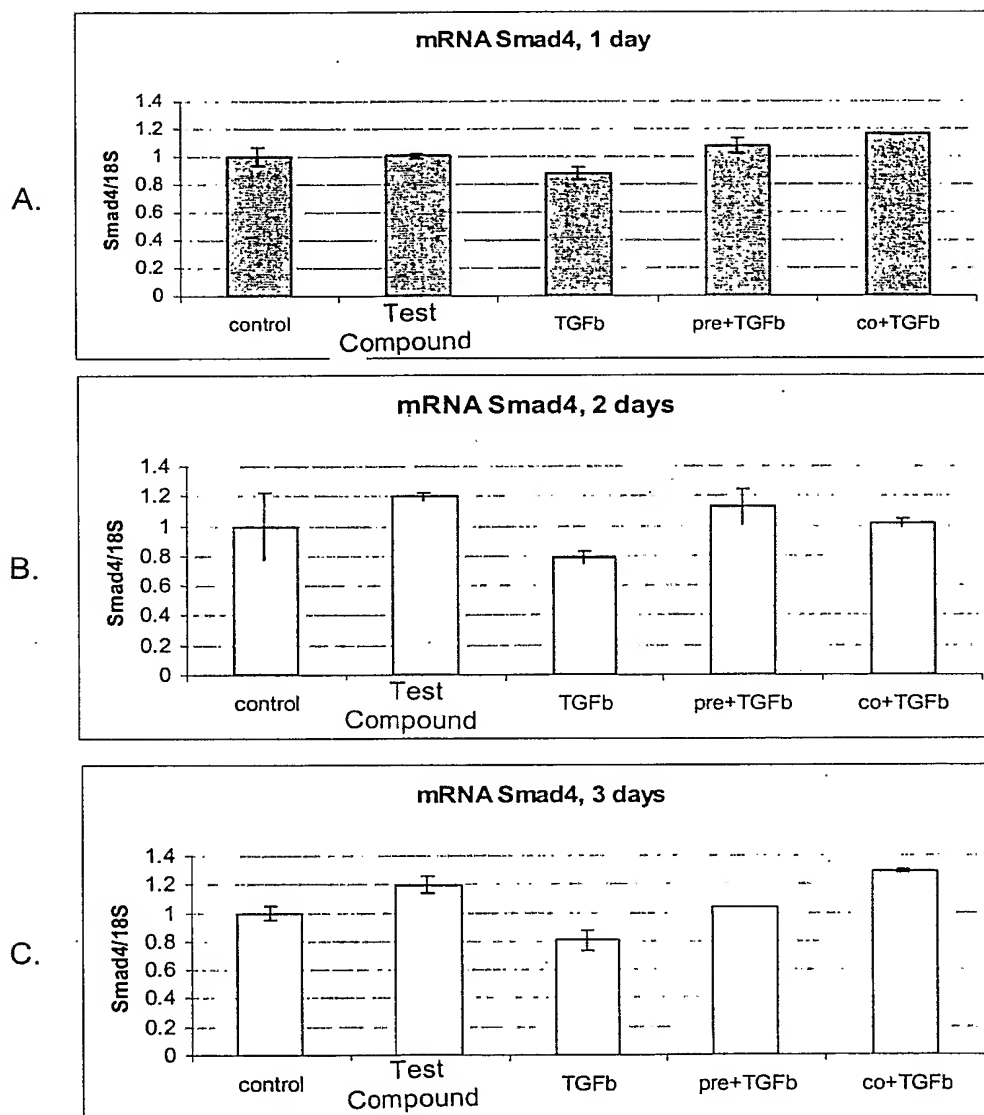
FIGURE 31

FIGURE 32

FIGURE 33

A.

mRNA Fibronectin, 1 day

B.

mRNA Fibronectin, 2 days

C.

mRNA Fibronectin, 3 days
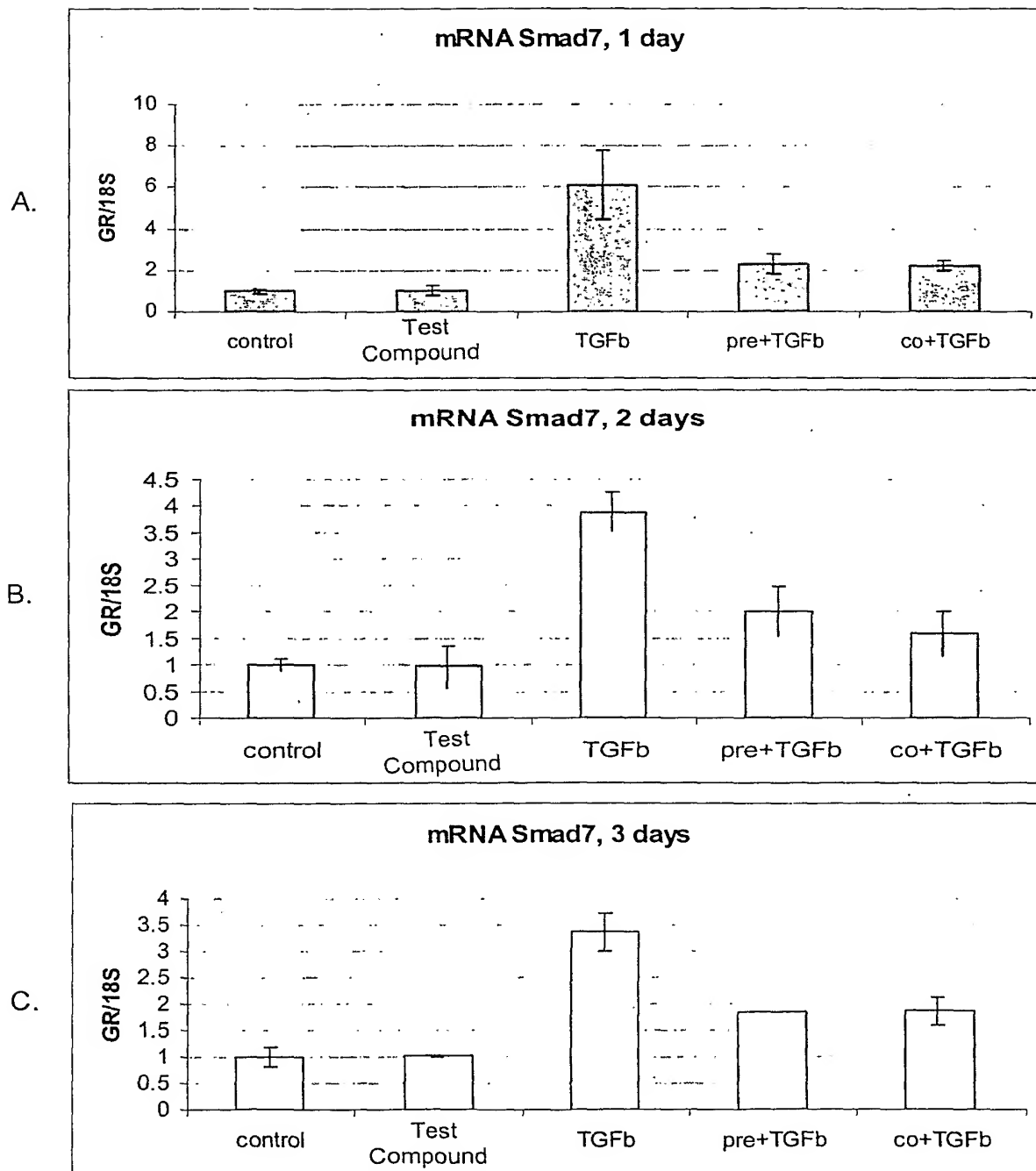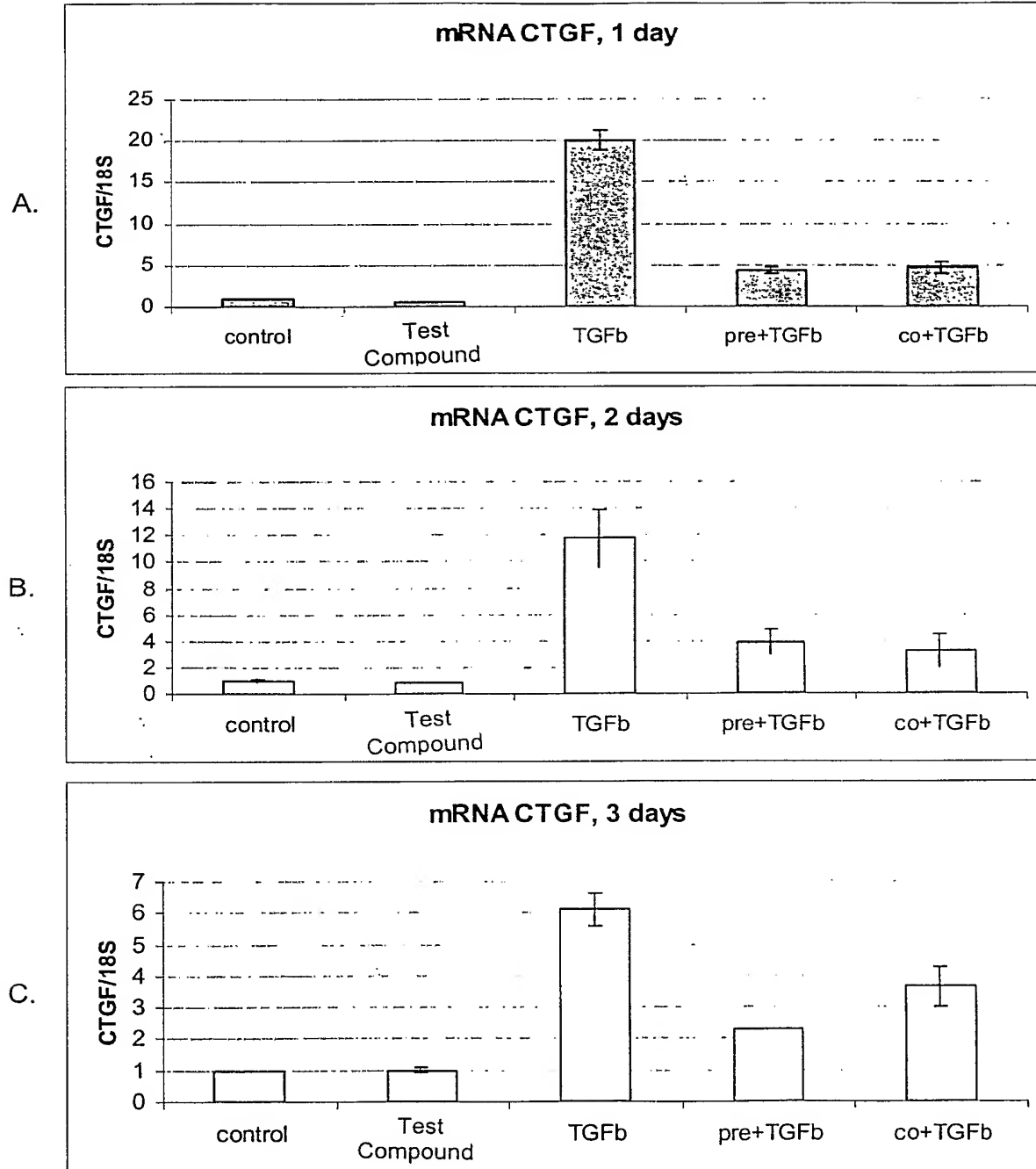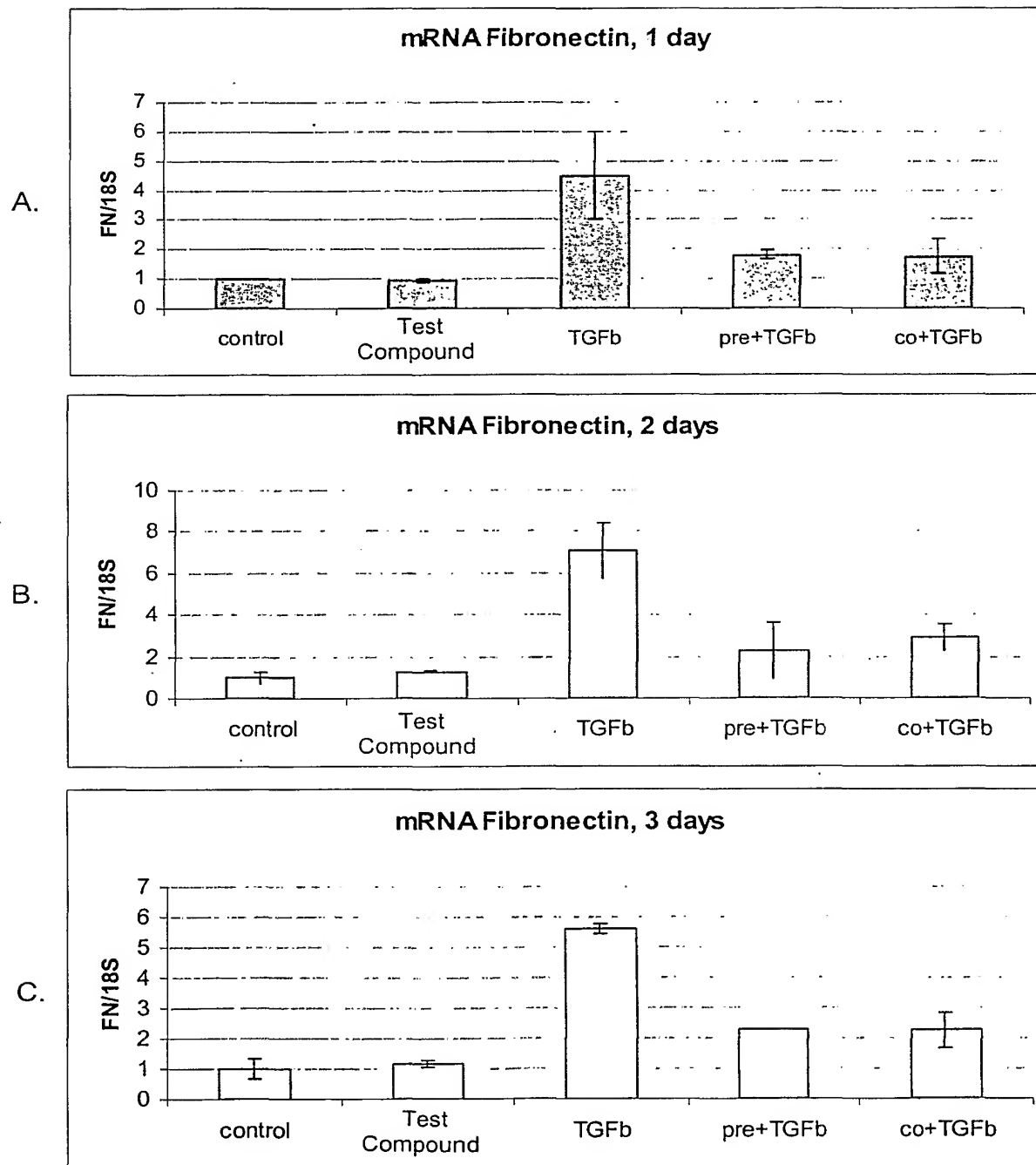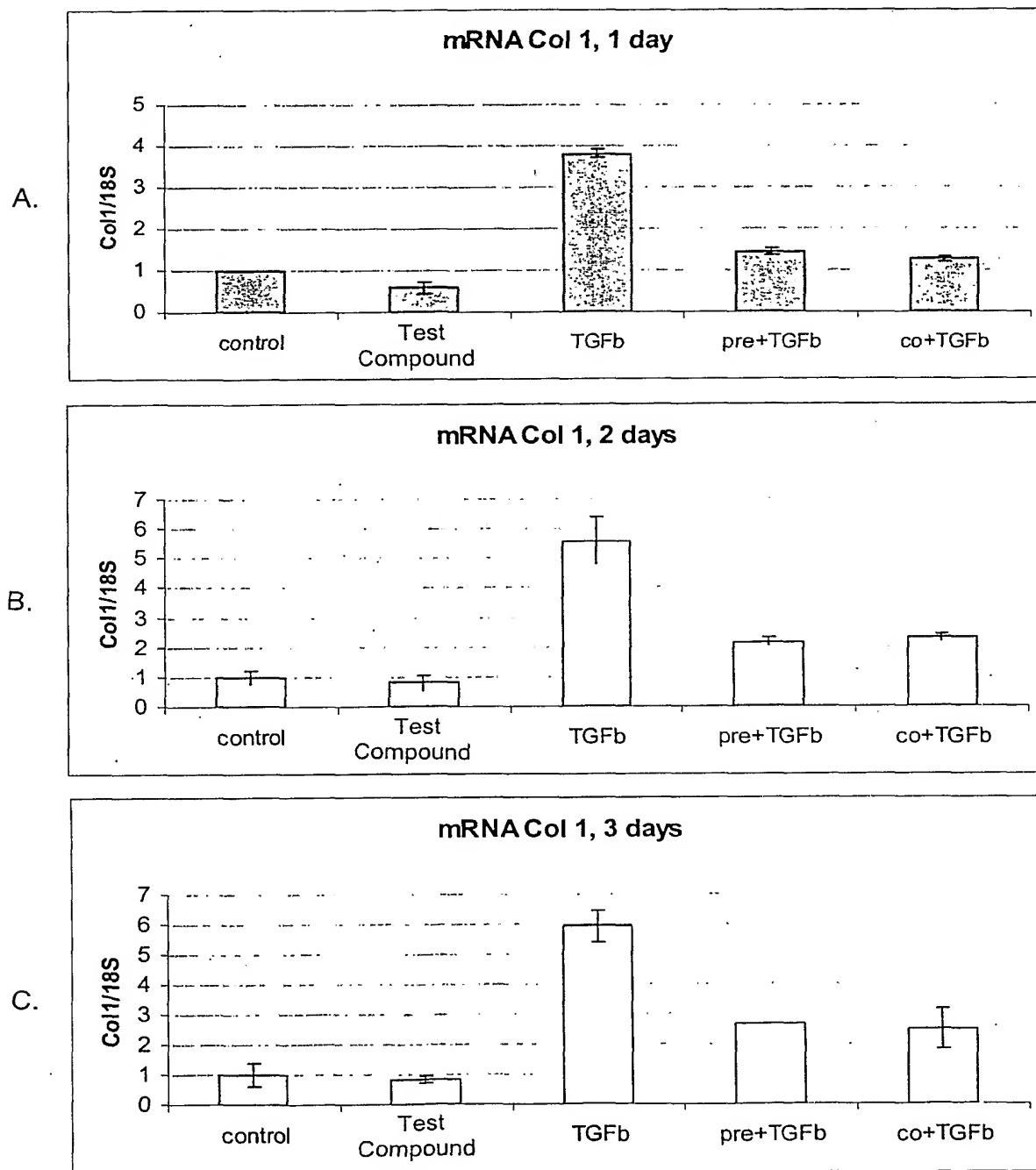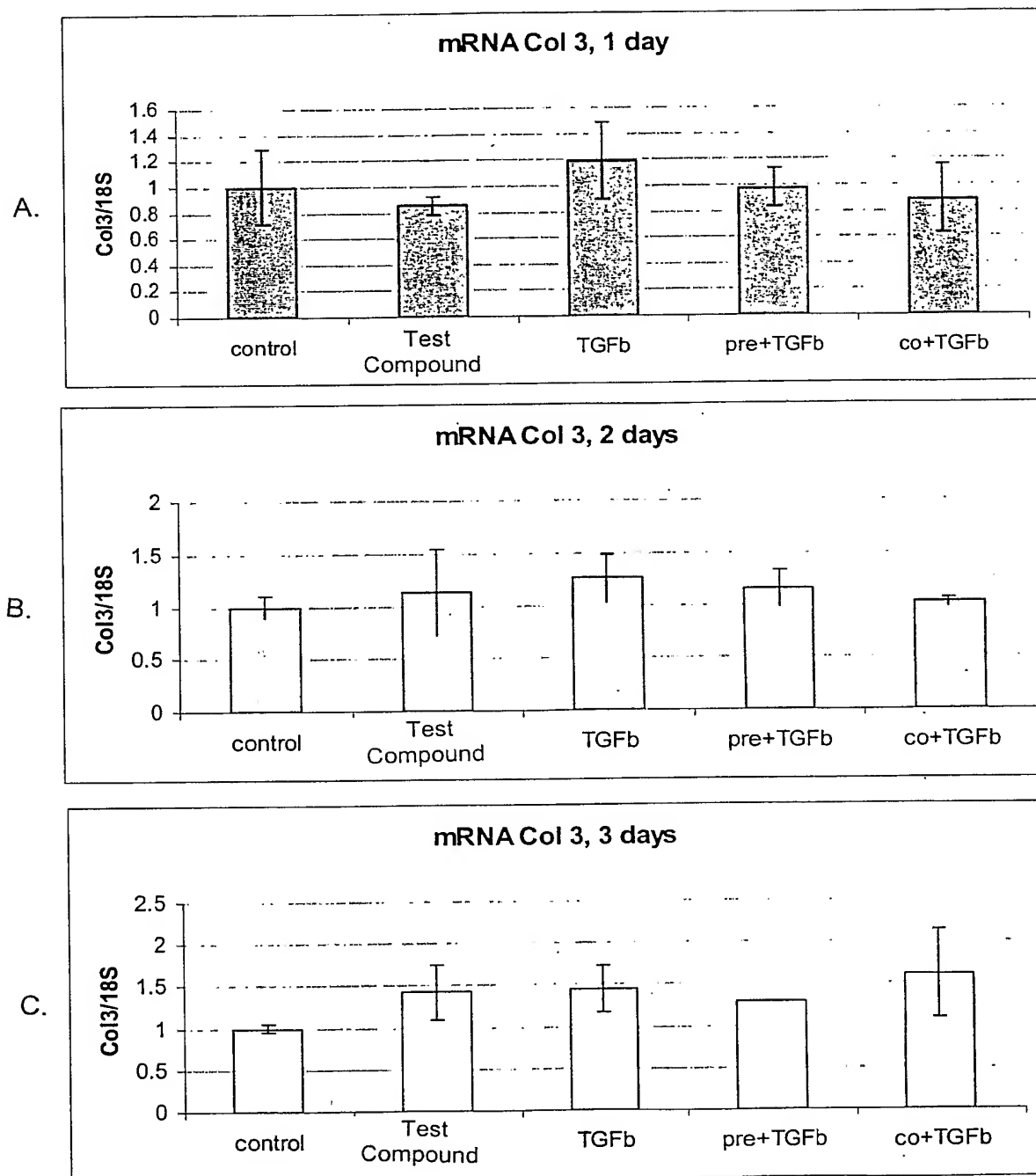
FIGURE 34

FIGURE 35

FIGURE 36

FIGURE 37

FIGURE 38

A.

mRNA TAK1, 1 day

(Bar chart, y-axis: TAK1/18S, 0 to 1.4; x-axis categories: control, Test Compound, TGFb, pre+TGFb, co+TGFb)

B.

mRNA TAK1, 2 days

(Bar chart, y-axis: TAK1/18S, 0 to 1.6; x-axis categories: control, Test Compound, TGFb, pre+TGFb, co+TGFb)

C.

mRNA TAK1, 3 days

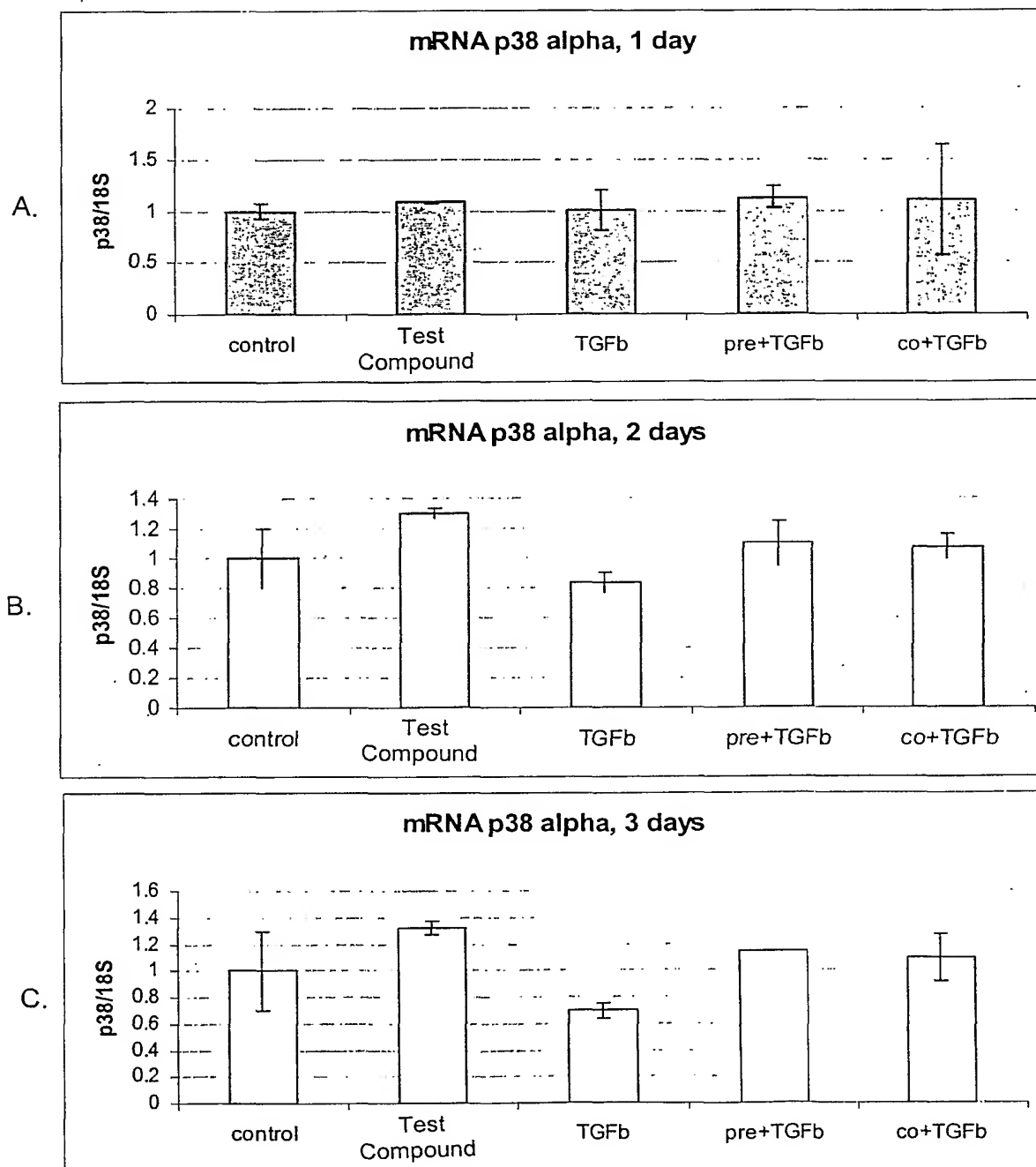*(Bar chart, y-axis: TAK1/18S, 0 to 1.6; x-axis categories: control, Test Compound, TGFb, pre+TGFb, co+TGFb)*
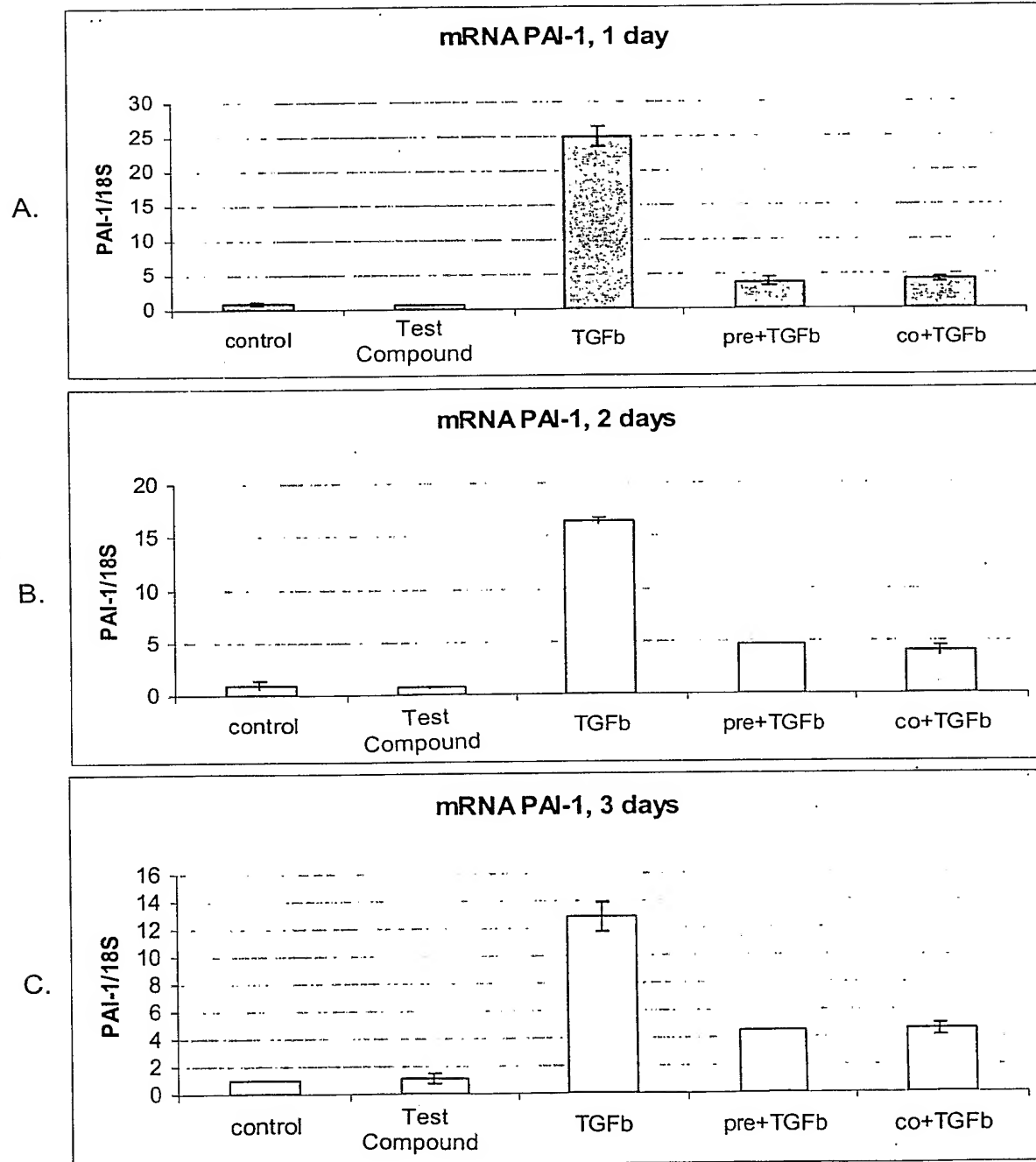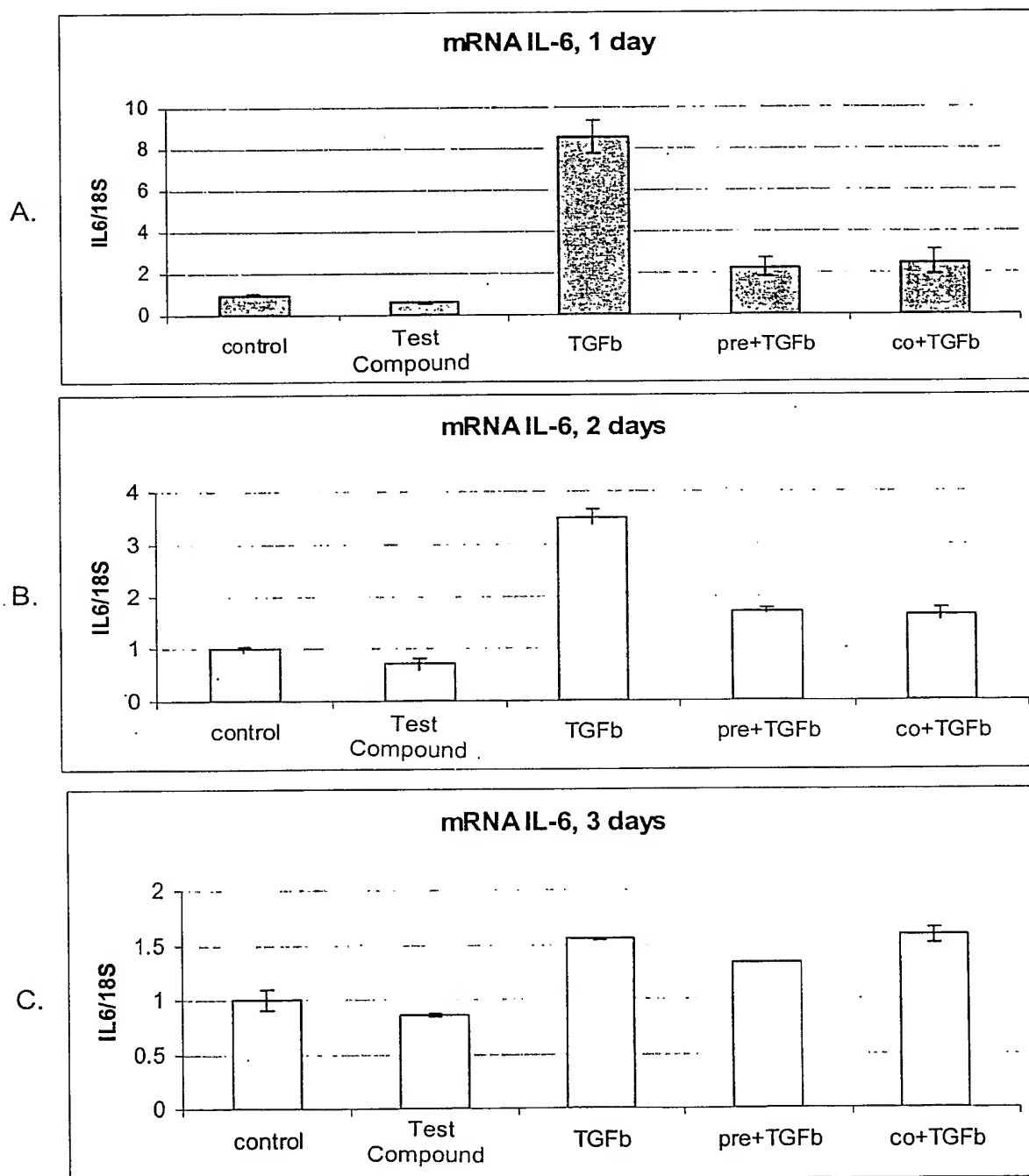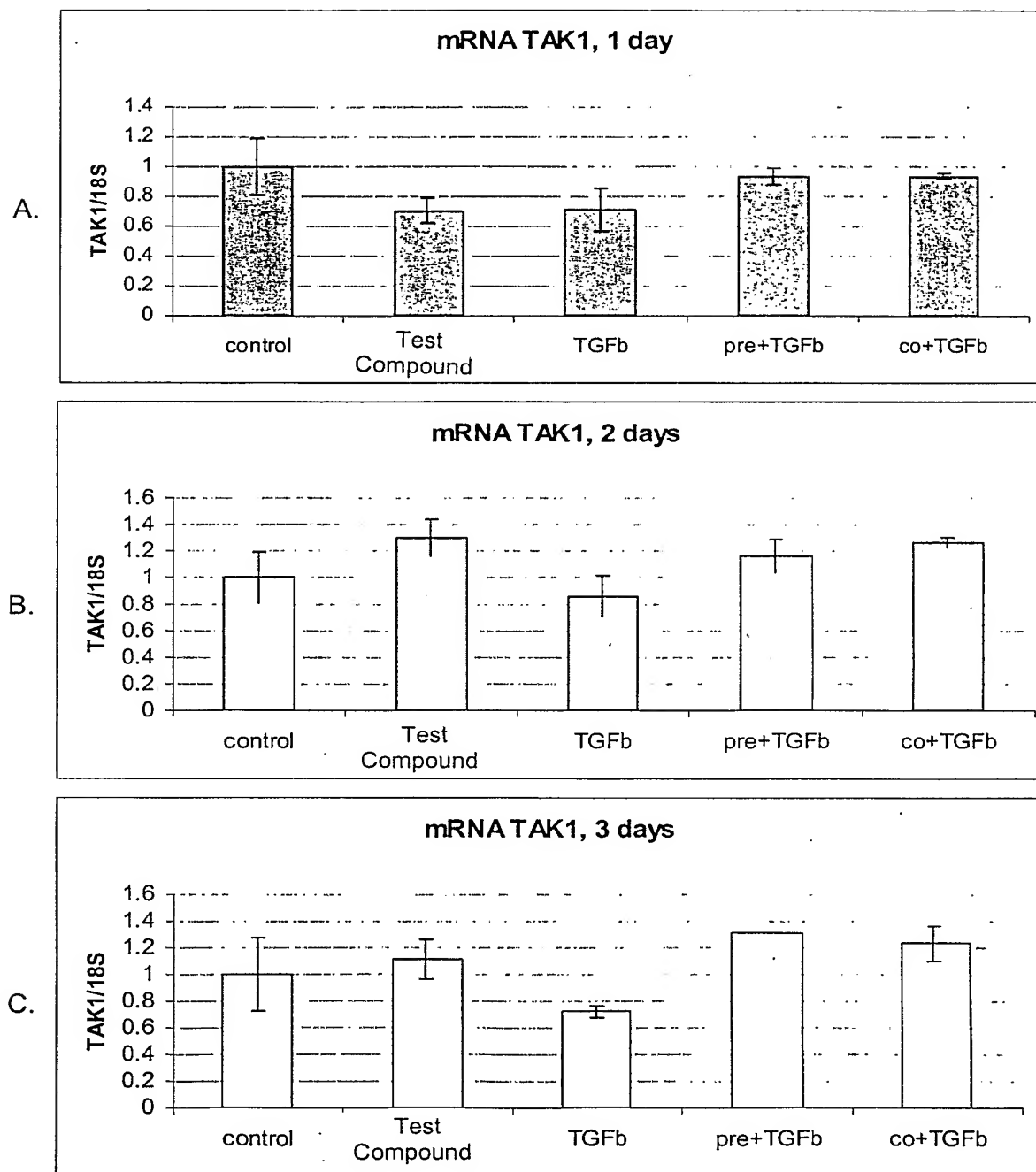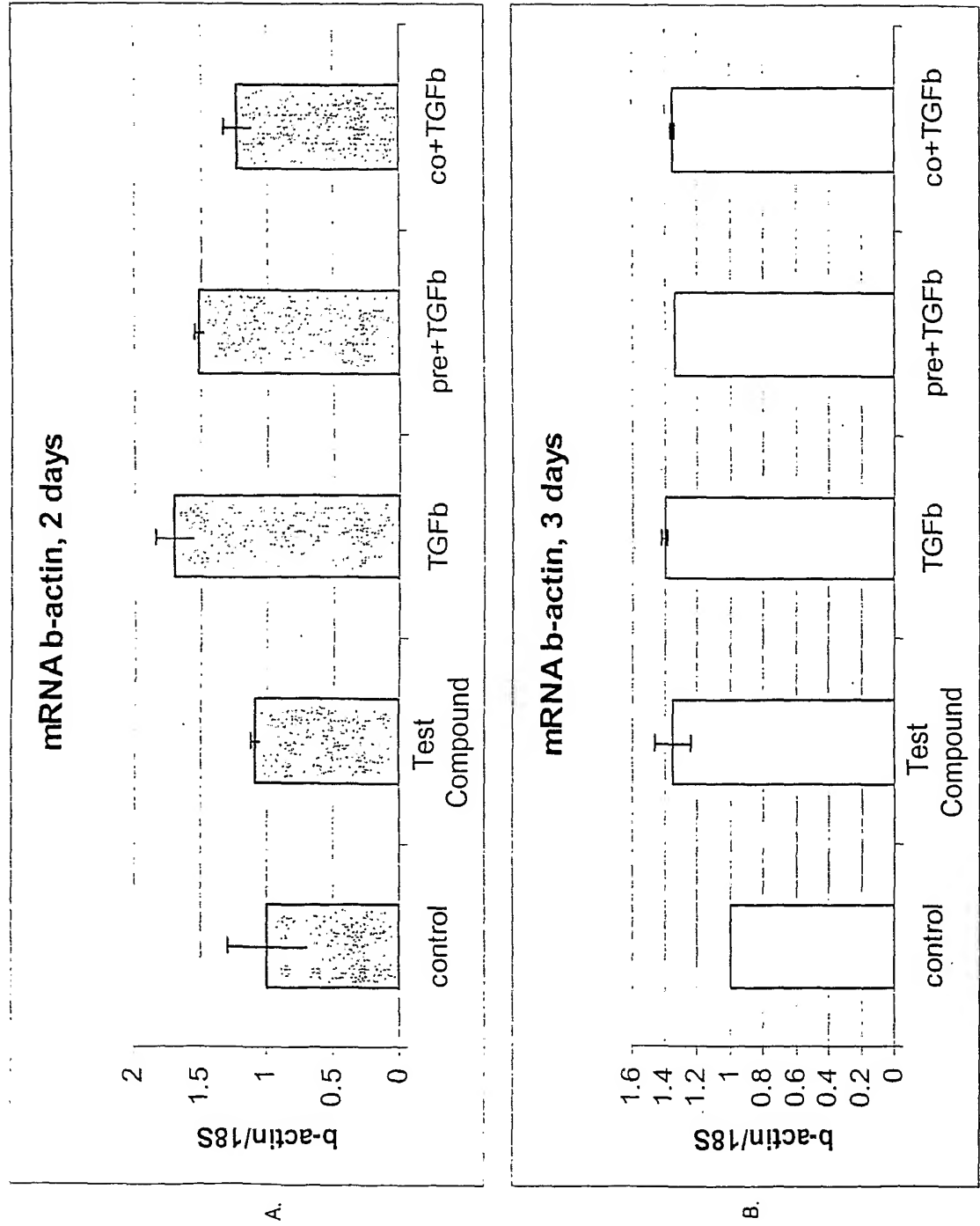
FIGURE 39

FIGURE 40

FIGURE 41

FIGURE 42

mRNA Cox2, 3 days
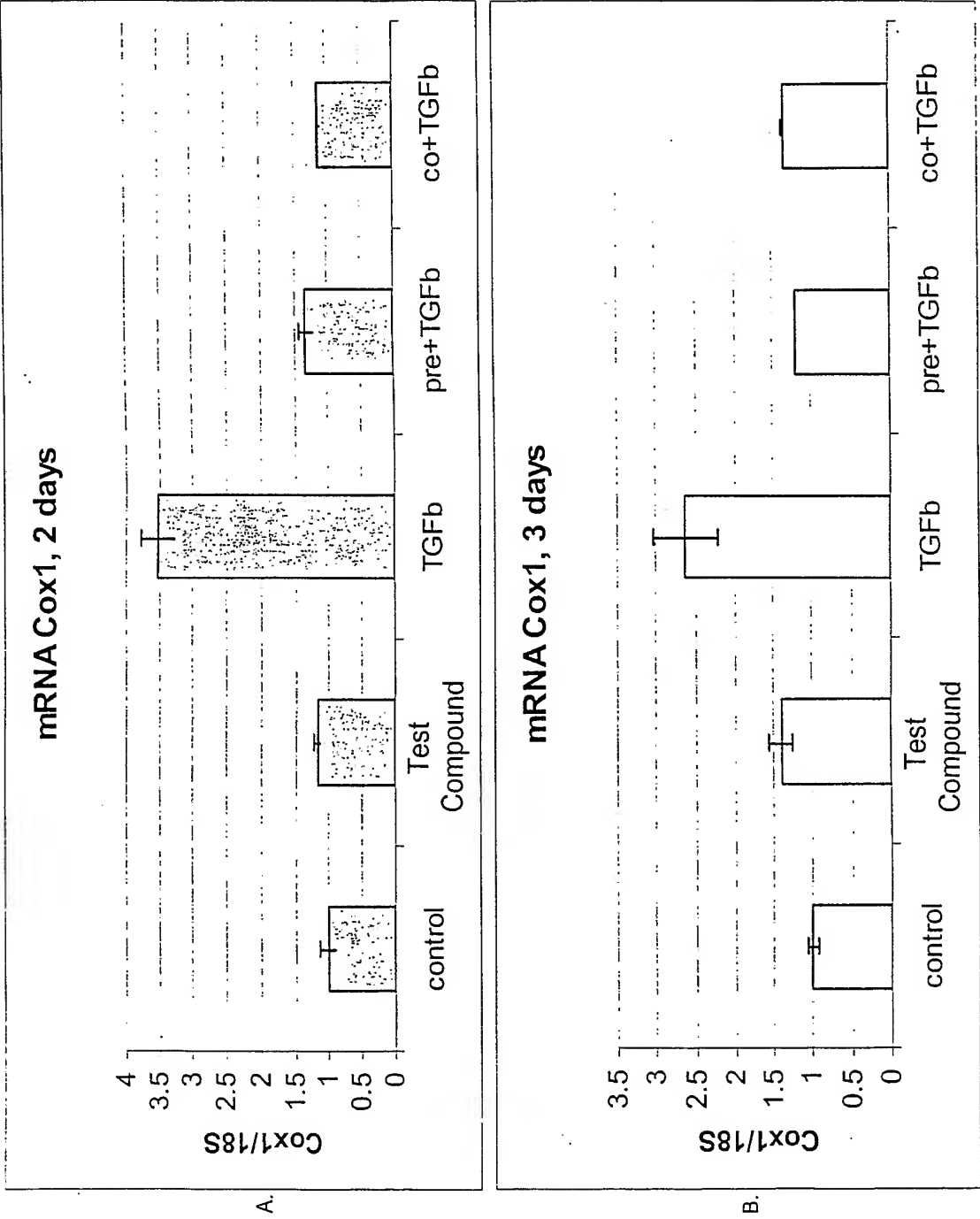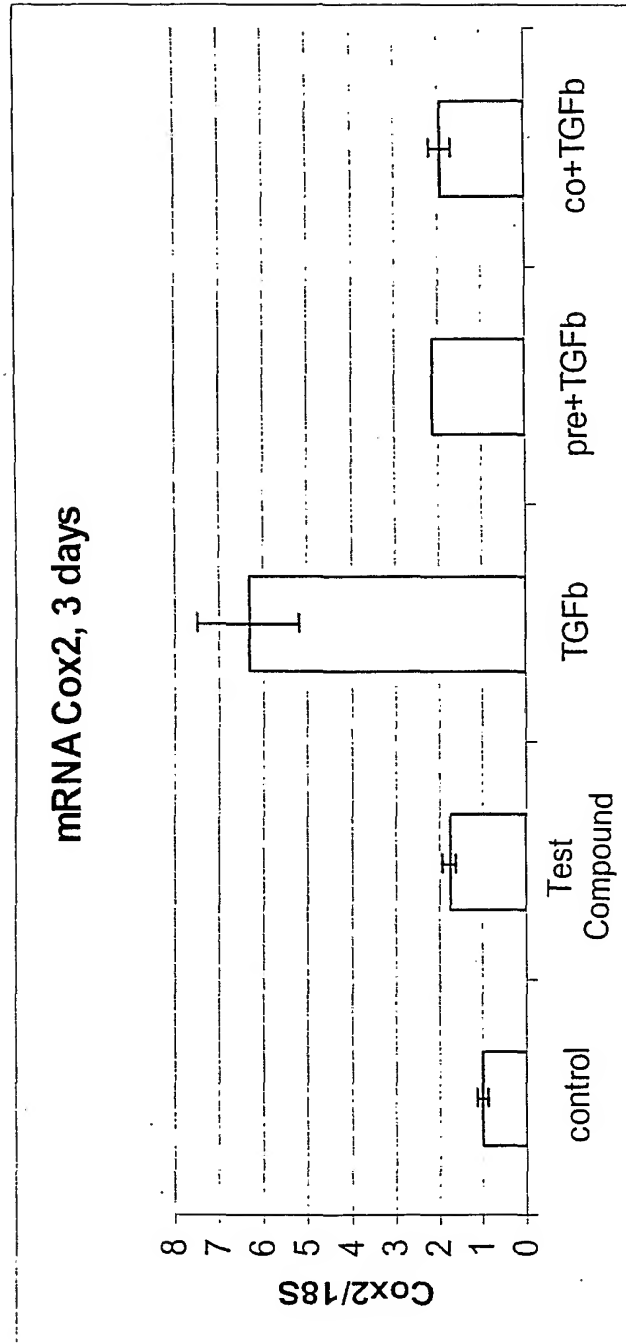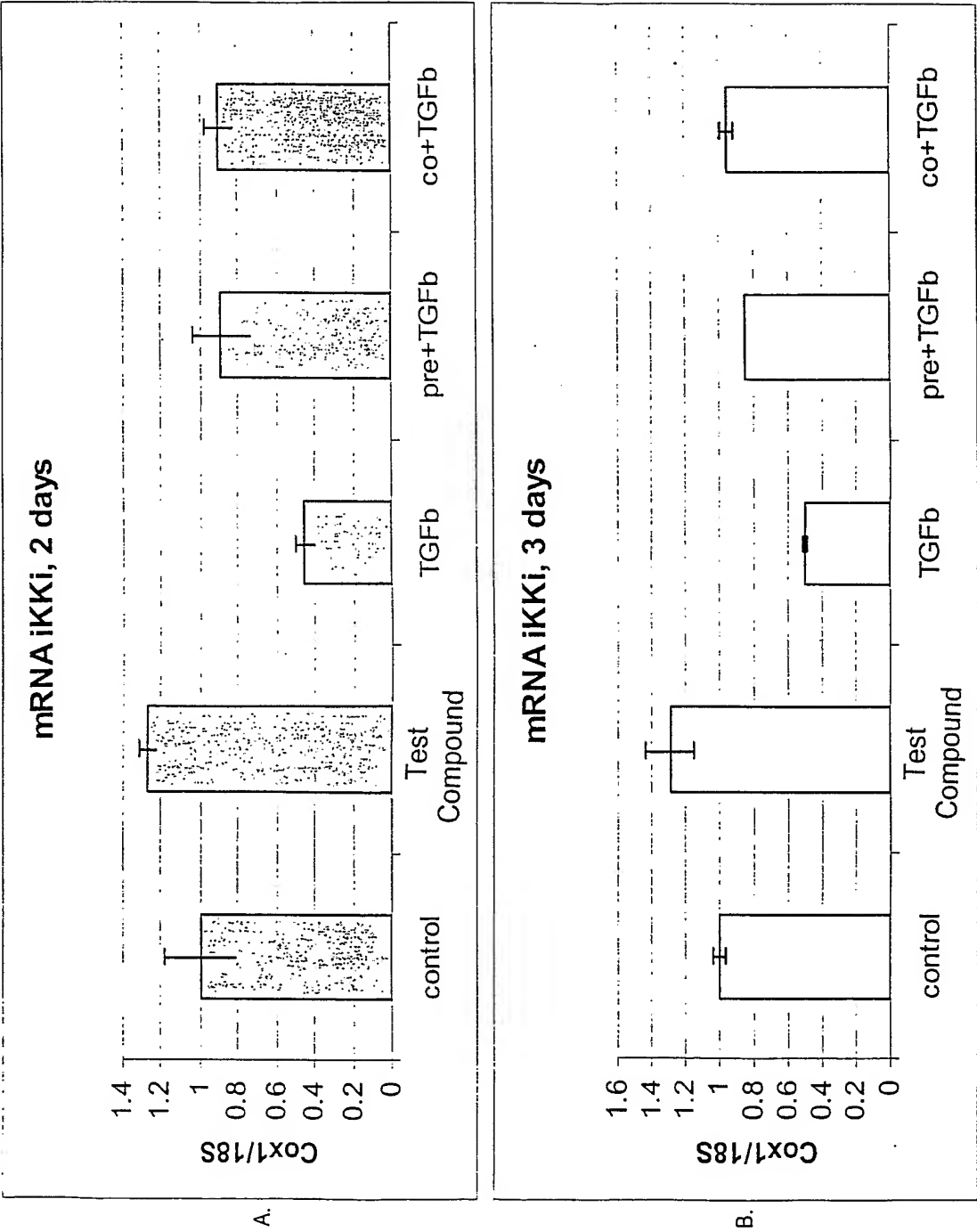
FIGURE 43

FIGURE 44

Percent Body Weight Change

Saline+1%MC+Saline
Bleo+1%MC+Saline
Bleo+1%MC+Dex
Bleo+Test Compound+Saline
Bleo+Test Compound+Dex
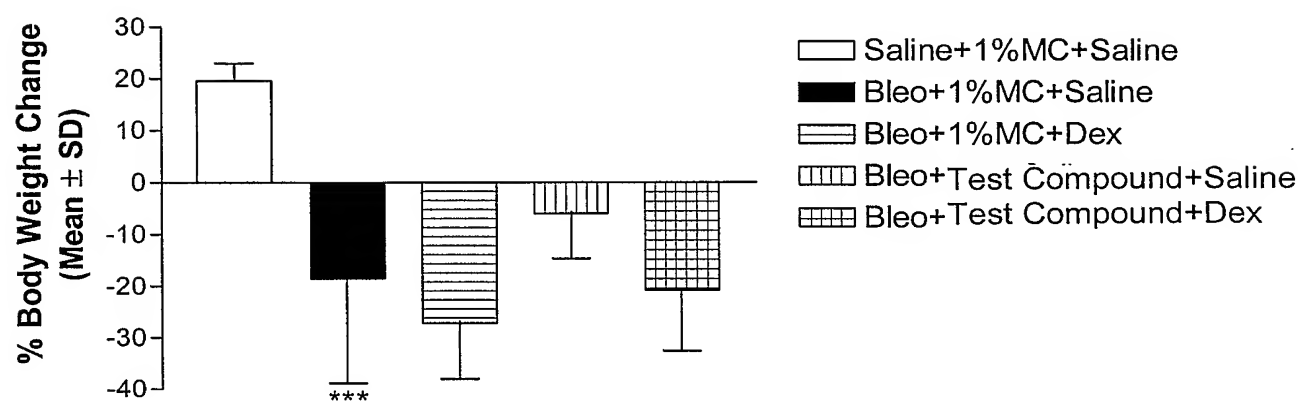
FIGURE 45

Total Hydroxyproline per Lung
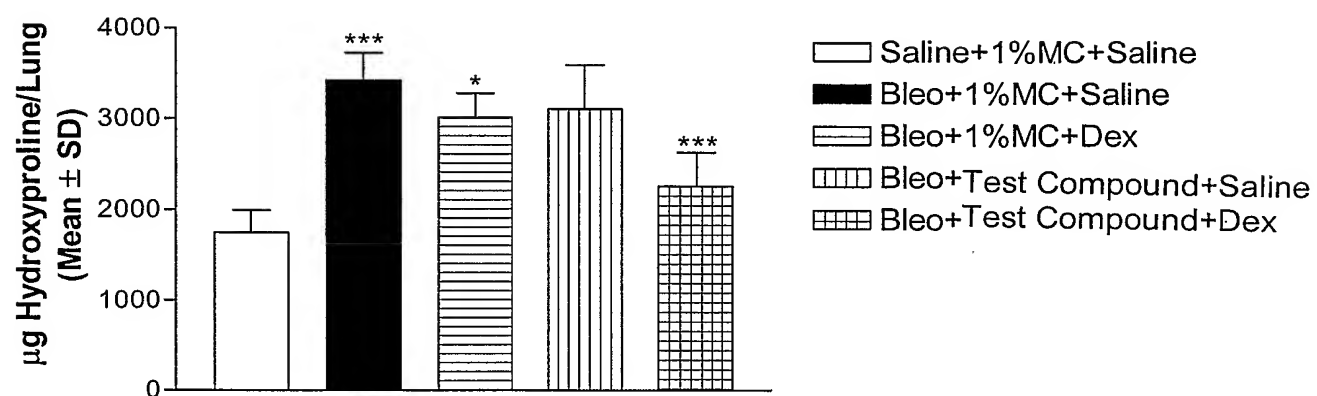
FIGURE 46

Lung Capacity
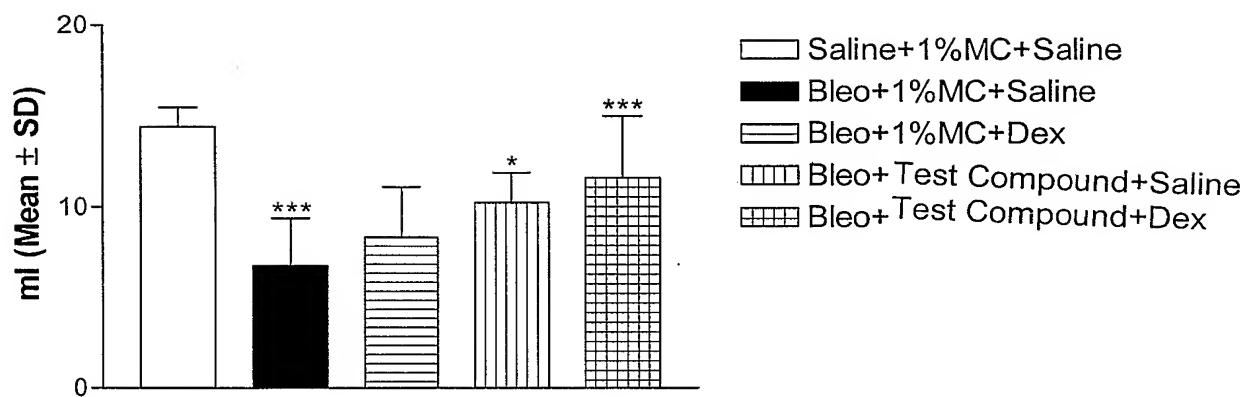
INTERNATIONAL SEARCH REPORT

A.	CLASSIFICATION OF SUBJECT MATTER
IPC(7)	: C07D 239/42, 239/94, 471/04
US CL	: 514/249, 279, 259, 252.17; 544/284, 393

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/249, 279, 259, 252.17; 544/284, 393

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6277989 B1 (CHAKRAVARTY et al) 21 August 2001 (21.08.2001), see abstract and full text.	1-12, 14-31, 33-44
Y		13, 32, 45
X	US 5,852,028 A (SUTO et al) 22 December 1998 (22.12.1998), see full text.	1-5, 13-24, 32-43, 45
Y		6-12, 25-31, 44
X	US 5,935,966 A (SUTO et al) 10 August 1999 (10.08.1999), see full text.	1-5, 13-24, 32-43, 45
Y		6-12, 25-31, 44
X	US 6,184,226 B1 (CHAKRAVARTY et al) 06 February 2001 (06.02.2001), see full text.	1-12, 14-31, 33-44
Y		13, 32, 45

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"P"	document published prior to the international filing date but later than the priority date claimed

"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 July 2003 (25.07.2003)	19 AUG 2003

Name and mailing address of the ISA/US	Authorized officer
Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450	Marianne Seidel
Facsimile No. (703)305-3230	Telephone No. 703-308-1235

Form PCT/ISA/210 (second sheet) (July 1998)

Continuation of B. FIELDS SEARCHED Item 3:
USPATFUL, CAS/STN ONLINE, REGISTRY, CAPLUS, MEDLINE
search terms: TGF-beta, p38-alpha, fibroproliferative, quinazoline